Designing and Implementing A Novel Multi-Parametric Bioreactor for The Functional Preservation of Vascularized Composite Allografts

by

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Abstract

Machine perfusion systems have been rapidly arising as an alternative to cold storage for organ preservation. Various pre-clinical and clinical trials have established the use of machine perfusion in extending preservation time and resuscitating organs procured from extended criteria donors. However, this technique has not been thoroughly explored for use with vascularized composite allografts (VCA). The purpose of this thesis was to incorporate subnormothermic (21°) machine perfusion into a novel multi-parametric bioreactor system for preserving abdominal wall grafts in a murine model. The bioreactor is a closed-environment chamber that provides electrical stimulation of the muscular component of the graft to improve function and prevent atrophy. It also allows for real-time monitoring of perfusate metabolites via in-line sensors to determine graft viability. Rat abdominal grafts were perfused for 12 hours with heparinized saline (HSa) or a modified histidine-tryptophan-ketoglutarate (HTK) solution. These grafts were compared to cold-stored and room-temperature stored grafts. Histopathological analysis during preliminary studies revealed that machine perfusion resulted in greater deterioration of grafts when compared to other storage techniques. It was concluded that, although the bioreactor system was successful in accessing graft vasculature and acquiring data from in-line sensors, the HSa and HTK solutions were not sufficient for graft preservation. Future experiments are necessary to determine additions to the perfusate for optimal graft preservation. Further work is needed to establish parameters of different metabolites to determine graft viability throughout the perfusion time. Electrical stimulation protocols must also be optimized. Efforts are on-going to finalize the bioreactor design and to develop a system that can adequately extend VCA viability.

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1. Introduction

Vascularized composite allotransplantation (VCA-Tx) is a procedure that involves transplanting vascularized composite allografts (VCA) as a functional unit. It can correct defects that cannot be reconstructed with autologous tissues. Examples of VCAs include facial and extremity transplants. VCA-Tx has the potential to treat thousands of individuals world-wide. In the United States alone, there are over two million amputees, with trauma accounting for the majority of these cases. VCA-Tx is a life-changing procedure that can improve quality of life by restoring function and aesthetics. However, a major barrier to the routine implementation of this surgical procedure is the preservation of allografts prior to transplantation. The gold standard for organ and VCA preservation is cold storage. However, VCAs are especially challenging to preserve with this method because of their composite nature. Each tissue type has different ischemic tolerance, with muscle being the most vulnerable to cold ischemic insult. Most grafts must be transplanted within four to ten hours of harvest in order to maintain viability of the muscle component. Machine perfusion systems with a wide range of variables, including temperature, perfusate, and flow regimens, have shown promise in clinical settings of solid organ preservation. Evidence from many animal and human studies suggest that machine perfusion can successfully preserve VCAs. The purpose of this project is to investigate the feasibility of extending VCA preservation time with a bioreactor system that can simulate physiologic conditions. The following narrative will explore the origins of machine perfusion as a method of organ preservation and the expanding efforts to establish this technology in the field of VCA-Tx. A murine abdominal wall transplantation model suited for this project will be detailed, followed

by the design and realization of a customized bioreactor chamber with electrical stimulation and metabolite sensing capabilities.

2. Overview of Literature

2.1 Solid Organ Ex-Vivo Machine Perfusion

2.1.1 Origins of research in ex-vivo solid organ machine perfusion

The idea of extracorporeal circulation can be traced back to the 19th century. The belief, as expressed by Julien Jean Le Gallois, was that any portion of the body could be maintained indefinitely as long as the function of the heart and lungs could be replaced [1]. This set into motion research efforts to determine the plausibility of his statement. Early experiments in the 1800s involved injection of blood, oxygenated by physical agitation, into dismembered limbs [1]. As ideas progressed and developed, primitive perfusion machines were constructed that could establish and control blood pressure and flow rates. Alexander Schmidt and Carl Ludwig were likely the first to incorporate a method of blood oxygenation to their perfusion circuits [2]. The goal of these perfusion experiments shifted from maintenance of the organ to understanding their physiological action after removal from the body. By doing so, researchers were able to solve many physiological problems in observing the organ or tissue outside of the body [2]. As more researchers became involved in the endeavor, closed circulation systems were developed and improved upon. An apparatus by Richards and Drinker allowed for the continuous oxygenation and perfusion of blood through isolated mammalian organs. Whereas previous iterations of such machines required repeated cessations of perfusion in order to oxygenate venous outflow, this apparatus made continuous perfusion possible [3]. They accomplished this by employing a dual pump system that simultaneously pumped blood through the organ under investigation and through an oxygenating device. However, these early devices had an inherent

limitation, in that they had to be manually powered. To bypass this challenge, electrically powered pumps began to appear in the literature, with the first electric rotary pump being described in 1913 [1]. As the foundations were laid for artificial circulation, physicians began to work on modifying the technology for use during cardiovascular procedures in humans. A limiting factor during correction of cardiac defects is the presence of blood in the surgical field, which makes it difficult for surgeons to clearly visualize defects and subsequently repair them. Through the combined efforts of scientists and physicians, perfusion systems were outfitted to bypass the heart and lungs, which served to render the heart dry for optimal surgical conditions. These efforts ultimately led to the development of the heart-lung machine as we know it today, one of the first instances of its use in a clinical setting being in 1951 [4]. This machine set the precedent for future systems that could safely and effectively perfuse tissues in a clinical setting.

2.1.2 Ex-vivo machine perfusion in solid organ transplantation

As organ transplantation became more and more established in the clinical setting in the 1960s, there was a need to revisit the original concept of Le Gallois: to preserve an organ once it had been removed from the body. Improvements in surgical technique resulted in transplant success rates that increased every year; as of 2016, one-year graft survival rates for kidney transplants are more than 90% [5]. However, one of the primary challenges of transplantation remains organ preservation. The first extracorporeal perfusion circuits required large amounts of autologous blood as the perfusate, which in many cases, was not sufficient from the animal subject itself. This often led to the supplementation with blood of other species, which understandably caused great harm to the perfused organ, and would not be feasible in a clinical setting [2]. In the late 1960s, Belzer and Collins began using a plasma derived perfusate for the hypothermic perfusion

of canine kidneys, with considerable success in transplantation [6]. However, the use of plasma-derived products presented the risk of spreading disease, and so there arose a need for a synthetic perfusate. Collins revolutionized organ preservation by developing a preservation solution that mimicked the intracellular environment of the kidney [6]. This perfusate, known as Collins' solution, led to the development of simple cold storage. This method involved flushing the blood from the organ with Collins' solution and storing at 4°C. Cold storage allowed renal preservation for 24-36 hours, and quickly surpassed the effectiveness of machine perfusion systems, which were greatly stunted by the technology available at the time [7]. The success and simplicity of this preservation method edged out burgeoning efforts to develop continuous perfusion machines [8]. Cold storage was more cost-effective and less technically challenging than machine perfusion. It became the most recognized preservation and storage technique, and persists to this day [6,7]. Despite the success of cold storage in preserving a variety of organs, evidence began to support the theory that injuries acquired during the preservation period could predispose the organ to rejection and result in transplant failure. A retrospective study of 215 liver transplants recipients in 1990 revealed a relationship between cold-ischemia time and postoperative primary graft function [9]. Under hypoxic conditions, cells switch to anaerobic metabolism, leading to a host of detrimental processes. Hydrogen ions begin to accumulate, decreasing cell pH and producing a large influx of sodium ions. Ischemia depletes cellular ATP, setting off a positive feedback loop that further impairs ATP production. These pathways, and many more, lead to disrupted cellular structures and eventual cell death [10]. Prompt reperfusion and oxygen delivery are essential to reverse tissue damage. However, organs subjected to periods of ischemia followed by oxygenated reperfusion often sustain ischemic-reperfusion injuries (IRI), a phenomenon that was first observed during canine cardiac experiments [10]. This umbrella term

encompasses a variety of complex and multi-faceted physio-pathological molecular cascades responsible for graft injury, including generation of reactive oxygen species (ROS), calcium overload, endothelial dysfunction, and enhanced inflammatory responses, among others [7,10]. The effects of reperfusion injury are so dramatic that cell death can continue for up to three days following the reperfusion phase [10]. It was evident that organ storage and preservation strategies had to be modified to mitigate the effects of hypoxia and IRI. Machine perfusion holds the potential to improve preservation by mimicking physiological conditions, and so there is much research into improving aspects of ex-vivo circulation, including the perfusate composition, the pump mechanism, and temperature. There were extensive efforts into creating a synthetic perfusate that could replace the functionality of blood and eliminate the challenges that come with it. Many solutions have emerged to suit the unique needs of different organs; these will be discussed in more detail in a later section. To mimic the action of the heart, double-acting pumps were introduced. These pumps establish a pulsatile flow that varies vessel diameter and movement of perfusate through them, more closely mimicking the natural flow of blood in the body [1]. Machine perfusion can support metabolism during preservation by providing oxygen and nutrients, which can reduce ischemia time. Viability testing has also evolved over the years and has proven to be an advantage over cold storage as it allows organs to be assessed for damage and to predict graft function prior to transplantation. For example, markers of dysfunction, such as aspartate aminotransferase (AST) for livers and glutathione-S-transferase for kidneys, can be evaluated and be used as predictors of function [11,12]. Temperature of perfusion is also an important factor in the support of an organ. At normothermic temperatures, organs experience more physiologic conditions, and it follows that preservation would be more successful. Studies have shown that perfusion at 37°C improves cellular energy, reduces tissue

injury and improves post-transplantation function in livers [12]. However, there is also evidence that subnormothermic perfusion can be similarly effective in preservation [13]. Overall, machine perfusion may hold the key to true organ banking practices, and there is much research going on to reach this ultimate goal.

2.1.3 Animal models of organ transplantation with machine perfusion

There have been countless animal models used to evaluate machine perfusion for organ transplantation. Canine, porcine, and murine models are among the most common. For brevity, only a few notable kidney, liver, and heart transplant studies will be discussed in this section.

Kidney transplantation. Several studies have shown the merit of machine perfusion over simple cold storage. Although kidney grafts fare particularly well with cold storage, machine perfusion can improve function in grafts obtained from donors after circulatory death (DCD) which undergo periods of warm ischemia. This is important because warm ischemia can only be tolerated for short amounts of time before irreversible damage occurs. A porcine study was able to salvage DCD kidneys that suffered over two hours of warm ischemia with machine perfusion. This could increase the donor pool and be more cost-effective than cold storage in the long term [14]. A rat kidney model evaluated the use of rewarming via oxygenated perfusion after 24 hours of cold storage at 4°C. Kidney viability was monitored by measuring injury biomarkers and renal function parameters in the perfusate and urine [15]. There were lower levels of injury markers (e.g. AST and lactate dehydrogenase) and higher parameters of function (e.g. sodium re-absorption and urine production) in rewarmed kidneys versus cold stored kidneys. Histology also showed less parenchymal and tubular injury, and better endothelial preservation [15]. Another

murine study mimicked the procurement of DCD kidneys and compared cold storage and normothermic and hypothermic perfusion with oxygenated blood prior to transplantation [16]. They found that hypothermic perfusion was superior to normothermic perfusion in that it preserved more renal structures and caused less release of ROS. However, it was evident that both normo- and hypothermic perfusion protocols were superior to cold storage alone [16]. Numerous porcine models have established the benefit of machine perfusion over cold storage in DCD models. Most studies subjected kidney grafts to machine perfusion after variable times of warm and cold ischemia, after which reperfusion was simulated by perfusing the organ with normothermic blood on an isolated perfusion system. Despite variability in perfusate, flow rate, temperature and pressure settings among these studies, all of them saw improved graft function in machine perfusion groups when compared to cold storage [17–23]. Non-transplant models assessed viability by measuring different biomarkers of injury and parameters of function. A renal autotransplantation model showed that hypothermic machine perfusion kidneys, when compared to cold storage kidneys, had less ROS production upon reperfusion, less proximal tubule damage (as assessed by brush border enzymes and lysosomal enzymes), and lower serum creatine levels [21]. Two studies utilizing a canine autotransplantation model assessed the effect of machine perfusion in restoring kidneys subjected to varying warm ischemia times. One study exposed kidneys to two hours of warm ischemia. Kidneys were either replanted immediately, hypothermically perfused for 18 hours, or subnormothermically (32°C) perfused for 18 hours. Kidneys perfused at 32°C showed life-sustaining function, with serum creatinine levels normalizing on post-operative day 9. However, the other two groups did not seem to recover from ischemic damage and were anuric posttransplant with resulting uremia [24]. The other study had similar results with warm perfusion, although the warm ischemia time was reduced to

30 minutes [25]. Both studies used acellular oxygenated solutions, which establishes synthetic perfusates as feasible options to recover renal function following warm ischemia. These studies involving DCD models demonstrate that kidneys can be pre-conditioned prior to transplant with machine perfusion to improve rates of graft function, particularly in marginal donors.

Liver transplantation. Applying machine perfusion to livers is challenging because it receives blood through two vessels: the portal vein and hepatic artery. However, there have been numerous studies and advances made in the preservation of livers, especially for extended criteria donors. A murine study found that machine perfusion of DCD livers at varying temperatures (4°C, 12ºC, 21ºC) provided superior liver function over cold storage and was comparable to non-DCD controls [13]. However, these grafts were perfused with a synthetic, acellular solution, and it was clear that an oxygen carrier was necessary to meet hepatocyte oxygen demand during higher temperatures of perfusion, since metabolic activity is increased. Another murine study using a perfusate with red blood cell concentrate as the oxygen carrier found that normothermic perfusion reduced bile duct injury and improved biliary epithelial function when compared to cold storage in DCD livers. Concentrations of biliary bicarbonate, reflecting biliary epithelial function, were significantly higher in machine preserved livers and comparable to non-DCD livers. A similar trend was found with biomarkers of bile duct injury [26]. Multiple porcine studies have also been carried out throughout the years. Porcine liver models more reliably predict human outcomes. Two studies observed the effect of one-hour warm ischemia time. Upon reperfusion to simulate transplantation, cold stored livers were non-viable with no bile production or glucose uptake and considerable necrosis. In contrast, machine preserved livers produced bile, had decreased alanine aminotransferase (ALT, the most sensitive

indictor of liver cell damage), increased ATP levels, and increased blood flow [27–30]. Even when warm ischemia is not a factor in liver procurement, machine preservation saw decreased ALT, increased bile production, and decreased reperfusion injury when compared to cold storage [27]. A severe complication unique to liver transplants is the occurrence of non-anastomotic biliary strictures (NAS), also termed ischemic-type biliary lesions. NAS can occur in as many as 30-50% of DCD liver transplants. Studies have shown that machine perfusion could have protective effects on the biliary tree and prevent NAS, which could expand the donor pool [31]. Animal models and data from discarded human livers provide some evidence that machine perfusion reduces bile duct injury, and restores regenerative capabilities of biliary epithelium [31]. A murine study sought to understand the mechanisms of recovery in order to allow more rational optimization of perfusion systems [32]. Fresh and DCD livers were subjected to normothermic machine perfusion for six hours and subsequently transplanted. Metabolic analyses of major pathways, including amino acid metabolism, glucose metabolism, oxygen uptake, and electron transport, were obtained. Results showed that livers were stable over the six hour machine perfusion period and warm ischemic livers recovered to a transplantable state after two hours [32]. These studies, and many more throughout the decade [30], provide evidence that supports the ability of oxygenated machine perfusion to restore livers that have undergone warm ischemia (i.e. DCD livers).

Heart transplantation. Cardiac transplantation faces the same challenges as other organ transplantations, namely, donor shortage. Extended criteria donors have the potential to increase the donor pool, but organs from these sources are often not eligible for transplant due to high risk of transplant failure. Several studies employing large animal models have shown that machine

perfusion can offer myocardial protection during the preservation phase [33–39]. When compared with cold storage, machine perfusion, regardless of temperature, produced hearts with decreased lactate levels, increased energetic metabolism, and increased contractility [34,35,37]. A study evaluated canine hearts after four hours of cold storage versus hypothermic machine perfusion, after which they were connected to a non-working heart Langendorff system. The Langendorff system allows monitoring of isolated hearts; many parameters can be analyzed in real time, including pressure, biopotentials, and heart rate [40]. They found that the cold stored hearts had intramyocardial acidosis and significant ATP depletion. In contrast, machine perfused hearts had less oxidative damage and cell apoptosis. Machine perfused hearts also had increased systolic and diastolic function. In another branch of this study, machine perfusion was able to reanimate canine hearts that had suffered one hour of warm ischemia. These hearts demonstrated full functional recovery upon connection to the Langendorff system, whereas cold stored hearts showed no systolic function [34]. The effect of machine perfusion on ideal donor hearts, i.e. with little to no warm ischemic time, was also evaluated. It was found that, in comparison to cold storage, machine perfused hearts had improved hemodynamics, decreased myocardial acidosis, increased energy storage and improved endothelial function [34]. Two studies transplanted preserved donor hearts in canines and pigs and were able to demonstrate superior function in machine perfused hearts [38,39]. One study preserved hearts for 14 hours with cold storage or retrograde perfusion through the coronary sinus. The hearts were then transplanted and evaluated over the course of 6 hours. All recipients receiving cold stored hearts required continued support on cardiopulmonary bypass and had increased creatine kinase release. Recipients receiving machine perfused hearts were able to be removed from cardiopulmonary bypass and pre-load recruitable stroke work was comparable to baseline values [38]. Another study used MR

spectroscopy and cardiac MRI to evaluate heart function after transplantation. They found that DCD hearts preserved with machine perfusion had almost complete metabolic recovery and replenishment of energy stores. The hearts also displayed excellent contractility following resuscitation with machine perfusion [38,39]. This finding confirms that machine perfusion can restore the viability of DCD hearts. However, these studies also discovered that machine perfusion is associated with myocardial edema, even at low perfusion pressures [33,36]. Fortunately, the occurrence of edema resolved in many cases after reperfusion on the Langendorff system and did not result in ventricle impairment. Overall, these studies suggest that machine perfusion has the potential to preserve donor hearts for long intervals and resuscitate hearts that have been damaged by warm ischemia.

2.1.4 Human clinical trials of organ transplantation with machine perfusion

The wealth of information provided by animal models has elucidated the potential of extracorporeal machine perfusion to improve post-transplantation outcomes, especially in extended criteria donors. These results have spurred interest in implementing machine perfusion in humans, which has resulted in numerous clinical trials that aim to compare outcomes between cold stored and machine perfused organs. Several devices, most tailored to a specific organ, have been developed for this purpose. The most common commercial machine perfusion systems used in these trials are illustrated in **Figure 1**.

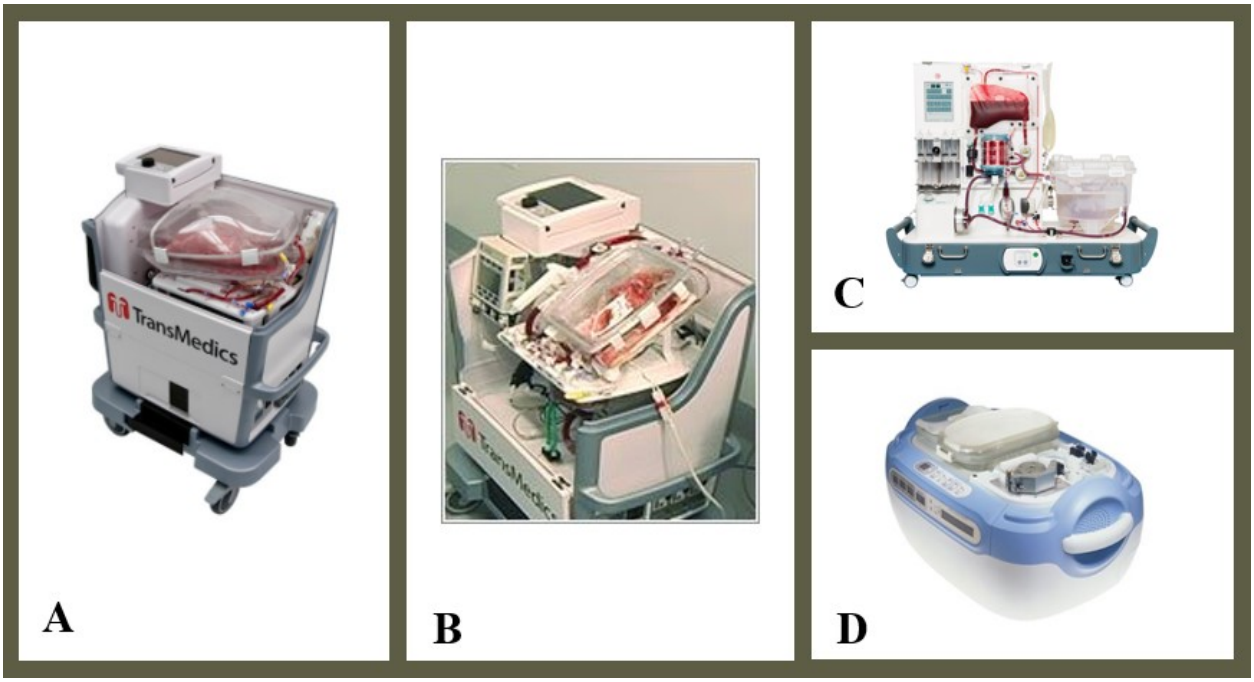

Figure 1. Most commonly used organ preservation systems. A) The OCS™ LUNG device B) The OCS™ HEART device C) The OrganOx metra® device for liver preservation D) The LifePort® Kidney Transporter

Kidney transplantation. Machine perfusion is occasionally used clinically for kidneys harvested from extended criteria donors or DCD. A pivotal study by Moers et. al. was the first large scale international, multi-center randomized control trial (RCT) to compare hypothermic machine perfusion and cold storage of kidneys [41]. The study involved 672 recipients: half received machine perfused kidneys, and the other half received cold stored kidneys. Machine perfused kidneys were preserved on the LifePort® Kidney Transporter device with University of Wisconsin (UW) solution at 1-8ºC. Machine perfused kidneys had a decreased risk of delayed graft function (DGF) and graft failure. The most striking result was an increase in 1-year and 3-year graft survival in the machine perfused group (**Fig. 2**) [41,42]. A retrospective study analyzed outcomes of 63 human clinical studies that compared machine perfusion and cold storage use in kidney transplantation. The majority of these studies employed hypothermic,

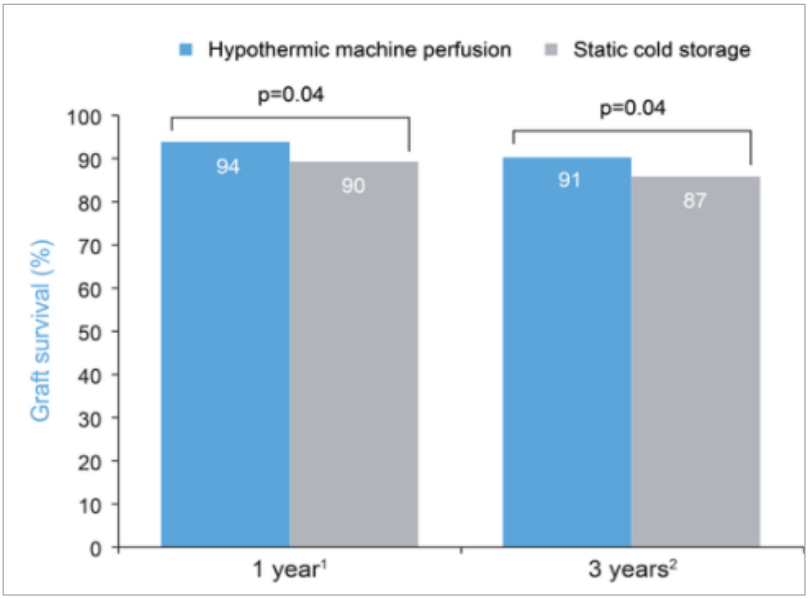

Figure 2. Increased kidney graft survival with machine perfusion [41, 42]

non-oxygenated machine perfusion with UW, although there is still no consensus on optimal oxygen and temperature settings during machine perfusion. Generally, machine perfused kidneys had extended hours of cold ischemia when compared to cold storage, yet saw decreased DGF rates in standard grafts and decreased primary non-function (PNF) rates in ECD grafts [43]. Although no statistical significance was reached, there was a strong trend towards decreased acute rejection in machine perfused kidneys [43]. A different approach found that there were no statistically significant differences between machine perfused and cold stored kidneys unless they were harvested from ECDs that were greater than 60 years old. A trend was also seen towards less dialysis time and lower short-term hospitalization costs in machine perfused kidneys [44].

Liver transplantation. One of the first clinical trials to compare machine perfused and cold stored livers was published in 2010. The study involved 20 recipients of machine perfused livers

matched with recipients of cold stored livers. The livers were hypothermically (4-6°C) perfused with an acellular perfusate. This study found trends towards decreased DGF, biliary complications, serum injury markers, and shorter hospital stays [45]. The same group followed a different cohort of patients, 18 in the machine perfusion group, and 15 in the cold storage group, to analyze the effect on IRI markers. Recipients of machine perfused livers displayed decreased levels of inflammatory cytokines (e.g. TNF-α, MIP-1α), HIF-1β (a hypoxia-induced marker), and adhesion molecules upon reperfusion [46]. These findings suggest that hypothermic machine perfusion can reduce proinflammatory cytokines, which often activate pathways related to IRI. A few studies involving normothermic perfusion used the OrganOx metra® device for liver preservation with promising outcomes. One study followed 20 recipients of machine perfused livers matched with control patients. 30 days post-transplant found decreased AST levels and a reduction in DGF, with 3 occurrences in the machine perfused group versus 9 in the control group, although this was not statistically significant [47]. An important finding is that normothermic perfusion requires the addition of an oxygen carrier to the perfusate to support higher metabolic function within the organ, whereas it is sufficient for the perfusate to be equilibrated with room air in hypothermic perfusion [30,45]. A more recent RCT study preserved 220 livers on the OrganOx metra® device. The authors found 50% lower levels of graft injury despite a preservation time that was 54% longer when compared to cold storage [48].

Heart transplantation. Instances of machine preservation in heart transplantation are fewer than with kidneys and livers. However, hypo- and normothermic perfusion systems have been developed for this purpose. Although hypothermic systems are not yet used clinically, the normothermic OCS™ HEART perfusion device has been used in clinical trials. One multicenter

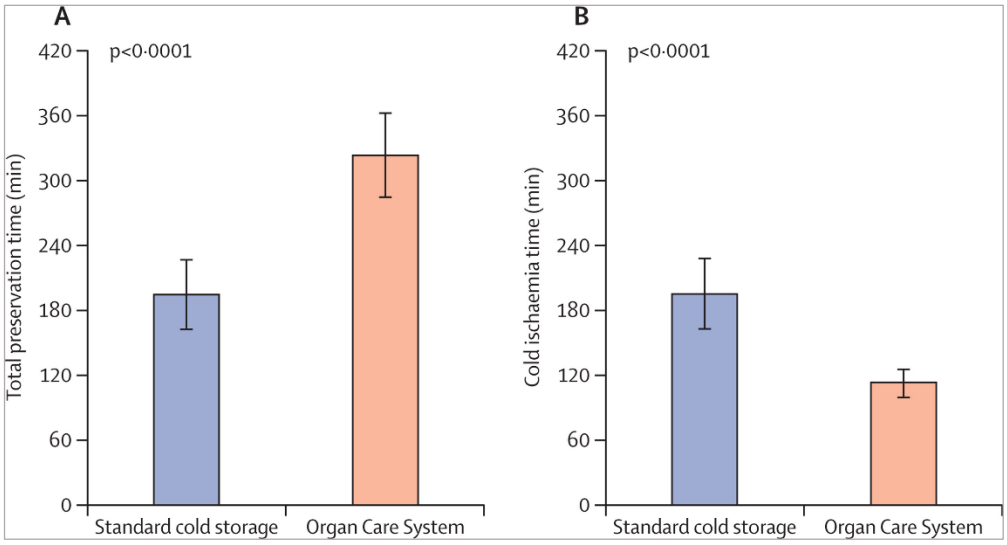

Figure 3. Mean out-of-body preservation times for heart grafts. A) Mean total preservation time B) Mean cold ischemia time [48]

RCT followed 67 patients in the OCS group and 63 patients in the cold storage group. 30-day patient and graft survival were similar in both groups and there were no significant differences in rates of rejection, length of ICU stay or cardiac related severe adverse events, despite longer preservation times in the OCS group (**Fig. 3**) [49]. However, outcomes suggested some advantages of machine perfusion over cold storage, namely reduced cold ischemia time (**Fig. 3**), improved ECD/DCD outcomes, and expanding time for distant graft procurement [50]. A small-scale trial in Australia involved three patients that received DCD hearts preserved on the OCS system. The hearts were subjected to less than 30 minutes of warm ischemia and preserved for an average of 254 minutes. All recipients showed favorable lactate uptake and normal cardiac function within one week of transplantation. Follow up at different post-operative days (176, 91, and 77 for each respective patient) showed good recovery [51]. Despite the lack of a control group and small group size, this trial demonstrated that machine perfusion can allow successful transplantation of DCD hearts. The overall trend within these few trials is that machine

perfusion, despite no clear advantage over cold storage of the ideal donor graft, can improve quality of ECD donors and subsequently expand the donor pool.

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Lung transplantation. Lung transplants are especially challenging procedures, not only because of the nature of the organ itself, but because over 80% of donor lugs are potentially injured and rejected by transplant teams [52]. Lungs are unique in that they need mechanical ventilation for optimal preservation. The OCS™ LUNG device was developed especially for this organ and has been used in several clinical trials. One study compared 20 high risk grafts (displaying pulmonary edema and poor deflation/inflation upon harvest) preserved on the OCS system with 116 standard, cold-stored grafts. Despite poorer quality of grafts in the OCS group and nearly doubled preservation times (653min vs 370min), these lungs had a partial pressure arterial oxygen:fraction inspired oxygen ratios (PO2:FIO2) that were comparable to control lungs [52]. There were no significant differences in secondary outcomes or 1 year survival rates between the two groups [52]. This suggests that machine perfusion enables successful transplants of high risk donor lungs. Another trial utilizing the OCS™ LUNG device compared 308 standard cold-stored lungs with 14 lungs harvested from donors that had a higher percentage of smokers [53]. Despite the higher risk of these grafts, recipients had excellent survival rates and superior early outcomes and 3 and 6 months post-transplant [53]. A study involving DCD lungs yielded similar results upon comparison of transplant outcomes between OCS perfusion and cold storage [54]. The first prospective, multi-center RCT in machine perfusion for lung transplants was completed in 2018. 151 patients received OCS perfused lungs and were compared with 169 cold-stored lung recipients [55]. The most compelling finding of this trial was a statistically significant decrease in risk of primary graft dysfunction grade 3 (PGDG3) in the first 72 hours post-transplant [55].

This suggests that machine perfusion can improve short-term outcomes following lung transplantation, but further studies would be needed to elucidate any long-term benefits.

2.2 Vascularized Composite Allograft Perfusion

Section 2.1 outlined the substantial success of solid organ transplantation and machine perfusion preservation. The improvement of surgical and preservation techniques ushered in by solid organ transplantation allowed VCA-Tx to become a possibility in the medical field. The potential of machine perfusion to improve solid organ preservation has sparked interest in applying this technology to VCAs.

2.2.1 The emergence of vascularized composite allografts

VCAs are transplanted as a functional unit that can correct defects that cannot be reconstructed with autologous tissues. As the name suggests, the feature that makes VCAs unique is the presence of different tissue types from the three germ layers [56]. This includes skin, muscle, tendon, nerve, bone and blood vessels. Other characteristics that distinguish VCAs from solid organ transplants include [56]:

- Transplanted to enhance quality of life and are not life-saving
- Are external, visible and are identifiable post-transplant
- Require nerve regeneration to regain function

The idea of extremity transplantations began to intrigue surgeons during World War II, but would not become a reality until many years later [57]. The applications of VCA-Tx can be clearly seen in military settings, where more than 1,500 American soldiers suffer traumatic limb amputations. Additionally, an estimated 700,000 civilians experience traumatic injury as the

second leading cause for extremity amputation [58]. The development of stronger immunosuppressant protocols in the 1990s led to successful transplant models in small and large animals [59]. Consequently, the first successful VCA-Tx was a hand transplant in 1998, and there have since been over 102 patients that have received upper extremity transplants world-wide [57]. Facial transplants are also becoming more common, with the first procedure occurring in 2005 and increasing to over 30 recipients world-wide [57]. Other milestone procedures include the first transfemoral lower extremity in 2011, an abdominal wall transplant in 2003, a penile transplant in 2006, and a uterus transplant in 2000 [60].

2.2.2 Challenges in VCA preservation

As with solid organ transplantation, the gold standard for VCA preservation is simple cold storage. However, VCA preservation is especially challenging because of the composite nature of the graft. Each tissue has different cold ischemic tolerance; for example, peripheral nerves and bone can tolerate up to 24 hours without detriment, while skeletal muscle and adipose tissue can deteriorate in as little as four [61,62]. Muscle is especially vulnerable to ischemic damage due to its high metabolic activity. Animal studies suggest that skeletal myocyte damage begins 3 hours post-ischemia and is often irreversible [63]. Due to this, it is likely that the muscle component will be the limiting factor in increasing preservation times. Establishing preservation time limits is further complicated by the variability of VCA and the nature of recipient defect, which causes procurement times to vary greatly—a facial allograft harvest could take well over 12 hours [64]. Although there is no established preservation time limit for VCAs, it is common to limit ischemia time to between 6-12 hours, after which risk of rejection may increase [64]. While machine perfusion could reduce ischemia time, and possibly increase overall preservation time, it

is important to consider the unique needs of VCA. Individual tissues would have different metabolic demands, which would require development of a specialized perfusate. It is also important to note that the muscle component of VCA may be difficult to preserve on a machine perfusion system. Injury patterns in skeletal muscle components of VCA have shown a similarity to those seen in cardiac muscle, which is particularly susceptible to edema when subjected to machine perfusion, as discussed in section 2.1.4 [33,36,60].

2.2.3 Animal models of VCA machine perfusion

As VCA-Tx is still in its infancy, there are yet no clinical trials of machine perfusion with VCAs. The feasibility of applying this technique must first be verified in animal models before it can be translated into the clinical setting. An early study established the feasibility of ex-vivo perfusion of normothermic blood in porcine forelimbs. Limbs were either machine perfused or cold stored for 12 hours. It was found that muscle stimulation was possible throughout the perfusion preservation, whereas there was a lack of response in the cold ischemic control limbs, and histology suggested preservation of the graft [65]. Another porcine study used vertical rectus abdominis muscle flaps as VCA models. 8 flaps were preserved subnormothermically (21°) for 14 hours, transplanted, and followed for 7 days. Compared to cold stored flaps, there was decreased IRI and post-transplant inflammation [66]. **Figure 4** illustrates the remarkable difference between perfused and cold stored grafts at POD7. A porcine autotransplantation model used myocutaneous flaps preserved for 7 hours with normothermic perfusion in a hyperbaric, hyper-oxygenated chamber. The grafts were viable at POD21 with no signs of necrosis and outcomes comparable to cold stored grafts that were only preserved for 3 hours (**Fig. 5**) [58]. One study saw positive outcomes 12 hours after transplantation of porcine

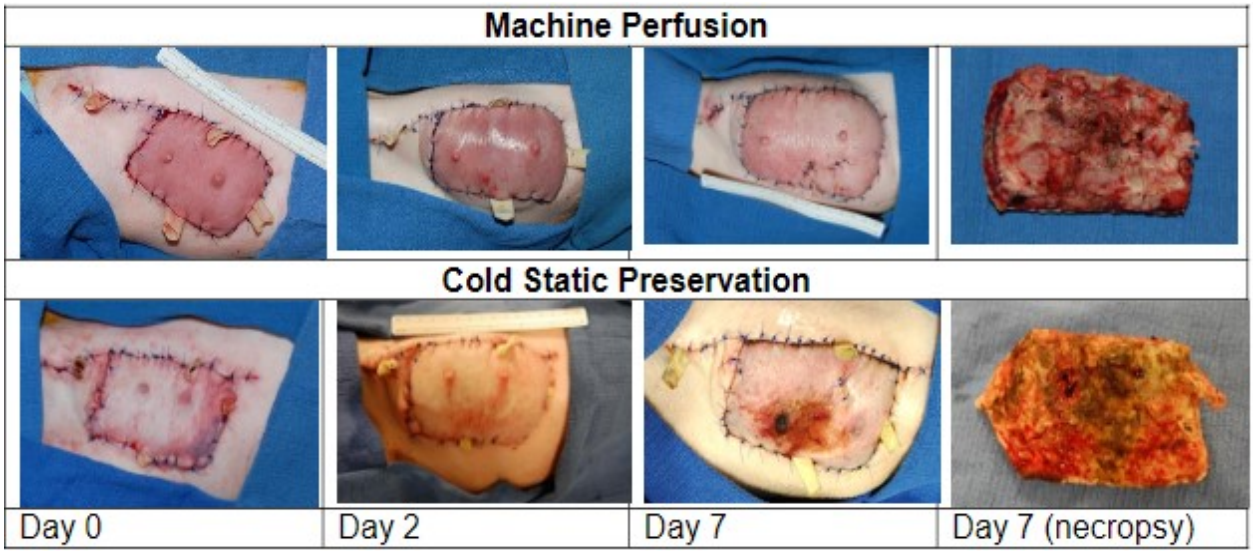

Figure 4. Representative images of machine perfused and cold stored VCAs at POD 0, 2, and 7. Cold stored grafts showed progressive signs of ischemic changes, including formation of ulcers around the nipples [65]

Figure 5. Representative images of gracilis myocutaneous flap auto-transplants. Normothermic perfusion produced viable grafts on POD21. Outcomes are comparable to grafts cold stored for 3 hours [57]

forelimbs that were preserved for 24 hours using normothermic machine perfusion with

autologous blood [67]. These limbs maintained neuromuscular electrical stimulation response, while cold stored limbs did not; single fiber muscle contractility was not significantly different in perfused limbs when compared to control contralateral limbs [67]. A recent study investigated the utility of subnormothermic oxygenated machine perfusion in a rat hindlimb transplantation model. Preliminary results showed that oxygen consumption was stable throughout the 2 hour preservation period along with decreasing lactate levels, which suggests restoration of metabolic activity [68]. All these studies strongly suggest that machine perfusion can extend the preservation time of VCAs past 4 hours with positive outcomes. Although more studies are needed to elucidate long-term effects of machine perfusion on VCA outcomes, the previously described results are encouraging.

2.3 Development of Synthetic Perfusate Solutions

2.3.1 Perfusate composition

Arguably, the most crucial component of successful ex-vivo machine perfusion is the composition of the perfusate that circulates through the tissue. An ideal synthetic solution would replace the function of blood. This endeavor has been the subject of many research efforts since the emergence of machine perfusion more than 100 years ago. Belzer and Collins solutions were among the first to be produced and made cold storage techniques possible. Many subsequent solutions were made with either Belzer or Collins as the base with additional components to meet certain needs, such as oxygenation. As seen in Table 1, the composition of preservation solutions can vary greatly. There is no established perfusate composition for organ

Table 1. Compositions of perfusates commonly used for organ preservation

Perfusate Solution Compositions							
Transmedics	**Belzer UW**	**Vasosol**	**Perfadex**	**AQIX RS-I**	**HTK**	**Celsior**	**Steen Solution**
Adenosine	Hydroxyethyl starch (50g)	Sodium gluconate (80mM)	Dextran (5%)	Sodium (135mM)	Sodium chlorida (15mM)	Mannitol (60mM)	Human serum albumin (25g/L)
Calcium Chloride dihydrate	Lactobionic acid (105mM)	Monopotassium phosphate (25mM)	Sodium (138mM)	Potassium (5mM)	Potassium chlorida (9mM)	Lactobionic acid (80mM)	Sodium caprylate
Glycine	Potassium dihydrogen phsophate (25mM)	Magnesium gluconate (5mM)	Potassium (6mM)	Magnesium (0.45mM)	Potassium hydrogen 2-ketoglutarate (1mM)	Glutamic acid (20mM)	N-acetyl-DL-tryptophan
L-Alanine	Magnesium sulfate heptahydrate (5mM)	Mannitol (30mM)	Magnesium (0.8mM)	Calcium (1.25mM)	Magnesium chloride (4mM)	Histidine (30mM)	Sodium
L-Arginine	Raffinose pentahydrate (30mM)	Adenine (5mM)	Chlorida (142mM)	Chlorida (119mM)	Histidine-HCl (18mM)	Calcium chloride (0.25mM)	
L-Aspartic Acid	Adenosine (5mM)	Calcium chloride (0.5mM)	Sulfate (0.8mM)	Bicarbonate (25mM)	Histidine (2mM)	Potassium chloride (15mM)	
L-Glutamic Acid	Allopurinol (1mM)	Ribose (5mM)	Glucose (5mM)	Organic acid BES (5mM)	Tryptophan (2mM)	Magnesium chloride (13mM)	
L-Histidine	Total glutathione (3mM)	HEPES (10mM)		Glucose (10mM)	Mannitol (30mM)	Sodium hydroxide (100mM)	
L-Isoleucine	Potassium hydroxide (100mM)	Dextrose (10mM)		Glycerol (0.11mM)	Calcium chloride-2H2O (0.015mM)	Reduced glutathione (3mM)	
L-Leucine		Pentastarch (50g)		Glutamate (300mM)			
L-Methionine		a-ketoglutarate		Glutamine (400mM)			
L-Phenylalanine		L-arginine		Aspartate (20uM)			
L-Proline		N-acetylcysteine		Carnitine (50uM)			
L-Serine		Nitroglycerin		Choline (10uM)			
L-Thereonine		Protaglandin E1		Thiamine pyrophosphate (40nM)			
L-Tryptophan				Human insuline (28mIU/L)			
L-Tyrosine							
L-Valine							
Lysine Acetate							
Magnesium Sulfate							

preservation solutions. In fact, a preclinical study with porcine kidneys showed conflicting results when different solutions were used for preservation [69]. Modifications to existing solutions are common, although establishing their efficacy is a matter of trail-and-error. However, the common goal of all solutions is to extend preservation times, and one of the main ways of doing this is to mitigate damage caused by hypoxic conditions and accompanying ATP depletion. Collins solution aims to preserve intracellular space composition upon ischemia. A high potassium content and glucose-based osmotic barrier was thought to achieve this but proved unsuccessful. The use of mannitol in place of glucose improved protection against prolonged ischemia. UW prevents edema with raffinose and lactobionate, mitigates ATP depletion by supplementing with a precursor of ATP (adenosine), and provides antioxidant properties with allopurinol. Histidine-tryptophan-ketoglutarate (HTK) contains histidine, a strong buffer, and mannitol, an osmotic barrier. Tryptophan and ketoglutarate are amino acids that help stabilize cell membranes. It is comparable to UW but has lower preservation costs [7].

2.3.2 Perfusate additions for improved graft function

Perfusion offers the unique opportunity of delivering therapeutic agents to VCAs prior to transplantation. This allows for the delivery of predetermined doses of therapeutic agents directly to the organ or graft and allow for subsequent monitoring This is especially important when optimizing tissues from extended criteria donors. Components can be added to the perfusate to maintain cellular integrity and vascular processes throughout the preservation time. Early attempts to do this yielded an acellular solution with a perfluorocarbon (PFC) emulsion. PFCs are inert solutions that have a great capacity for dissolving oxygen. A more recent addition is Hemarina-M101, an extracellular hemoglobin derived from marine invertebrates. It has a high oxygen affinity with antioxidant properties that could enhance perfusion preservation [22].

Altering perfusion conditions could enhance expression of protective proteins. The heme analog cobalt protoporphyrin upregulates protective genes that can reduce free radical injury and have anti-inflammatory effects. Carbon monoxide is another example; it is a potent vasodilator that can promote flow and oxygenation during perfusion [22]. Cell-based gene therapies and stem cells could also be applied to perfusion techniques, which could achieve immunomodulation to reduce rejection or enhance tissue functionality [62]. Preliminary studies have shown that allografts can be transduced with CTLA4-Ig, a costimulatory blocker, to promote graft survival in a variety of tissue types [62]. Perfusing allografts with a solution containing an adenovirus to increase expression of an anti-oxidative enzyme in endothelial cells was shown to mitigate transplant related vasculopathy [62]. Another study aimed to alter the physiologic properties of allografts to imbue it with therapeutic properties. A mouse adipose graft was perfused with adenoviral vectors containing anti-tumor cytokines or anti-microbial peptides. After perfusion, the graft was inset over a recipient with exogenously delivered tumor or bacterial cells. They found reduced tumor growth and bacterial cell counts. Additionally, the authors found minimal systemic adenoviral dissemination and associated toxicity [62]. Another application involves delivery of stem cells, such as mesenchymal stem cells, to the graft which could potentially support survival via immunomodulation. It is clear that the importance of the perfusate and its components cannot be understated. A perfusate developed for the specific needs of an organ or graft can improve post-transplant outcomes and function, which is imperative for patient survival and well-being.

2.4 Muscle Electrical Stimulation

2.4.1 Implementation and possible benefits

Electrical stimulation has mainly been studied as a method to prevent atrophy of denervated muscle by tapping into the plasticity of the nervous system. Approaches such as neuromuscular electrical stimulation (NMES) involve stimulating nerves to induce functional movement of associated muscles [70]. This therapy can improve muscle strength and improve recovery rates in patients suffering from spinal cord injuries and has been shown to be beneficial in human subjects [70,71]. This method has also been useful in preserving different muscle groups in critically ill patients with little to no mobility [72,73]. It has also been suggested that electrical stimulation can reduce capillary regression and muscle loss in denervated rat hindlimbs [74]. Most of these protocols involve specialized circuit designs, including H-bridges with biphasic stimulus capabilities, and implantable stimulation electrodes. Modifications can be made to stimulate muscles remotely and minimize invasiveness of the procedure. Although the use of electrical muscle stimulation has not been applied to VCA preservation, plenty of evidence suggests that stimulation of the muscle could result in better functional post-transplant outcomes. The addition of this therapy to VCA preservation has the potential of maintaining the volume of the muscle component during extended preservation times. Electrical stimulation would provide therapeutic movements in what would otherwise be an inert environment conducive to muscle atrophy.

3. Experimental Methods and Materials

3.1 Abdominal Wall Recovery and Transplantation Procedures

The murine abdominal wall transplant model is based on a procedure previously described by Broyles et. al [75].. Adult Lewis rats were housed with rat chow and water ad libitum, and maintained in accordance with institutional animal care and use committee guidelines. An illustrated representation of the complete procedure can be found in Appendix A.

Abdominal wall recovery. Rats were placed in an induction chamber with 2.5% isoflurane. Once the animal was anesthetized, it was placed on a surgical board in the supine position with isoflurane delivered at 2-2.5% through a nose cone. Depth of anesthesia was evaluated by employing the toe-pinch reflex test. The reflex had to be absent before proceeding. Respiration was also monitored regularly and ensured to be regular with thoracic and abdominal components. The abdominal area was shaved and disinfected with alternating applications of betadine and 70% alcohol. An area of the abdomen of approximately 4x4 cm was marked on either side of the linea alba. An incision was made at the inguinal canal and underlying fascia and fat were dissected to access the femoral and superficial epigastric vessels. Once these vessels were identified, the skin was incised around the previously determined perimeter. The first incision of the abdominal muscle was made at the linea alba. Bipolar cautery forceps were used to cauterize and cut the perimeter of the abdominal wall flap. The flap was then everted caudally, and internal organs were retracted to have a clear visual field. The external iliac vessels were identified and dissected from the bifurcation of the common iliac to just distal of the origin of the superficial epigastric vessels. All arterial and venous branches along the length of the main vessels were cauterized and divided. The inguinal ligament was cut, and the external iliac vessels were

divided at their origin. The abdominal flap was then flushed with 10mL heparinized saline (HSa) to remove blood and cool the flap to room temperature. Depending on the experimental group, the flap was either transplanted, after approximately 1 hour of ischemia time, or connected to the machine perfusion circuit for preservation experiments. Prior to transplantation, the proximal ends of the external iliac vessels were prepared for suture-free vascular anastomosis using a cuff technique. The vessels were pulled through 22-gauge polyethylene cuffs, the ends everted over them and tied circumferentially with 10-0 silk sutures.

Abdominal wall transplantation. Recipient animals were prepared in a similar fashion as the donors. The graft size was outlined on the abdomen and an incision made in the inguinal canal to expose the femoral vessels. The vessels were dissected, and any branches cauterized. They were then occluded with vessels clamps at their origin near the inguinal ligament. To preserve as much vessel length as possible, the femoral vessels were ligated and divided proximal to the origin of the superficial epigastric vessels. The vessels were irrigated with HSa. Vascular anastomoses were initiated by approximating the ends of donor external iliac vessels and recipient femoral vessels with a micro-vessel Acland clamp. The recipient's vessels were pulled over the cuffs on the donor vessels and secured with circumferential ties. The vessel clamps were then removed, and blood flow was reestablished. Only a few minutes were necessary to visually observe the reperfusion of the grafts and a return to normal color. The graft was then carefully placed over the recipient's abdomen to ensure the correct size and placement of incisions. Once this was established, the recipient skin and abdominal wall was removed. The donor graft was inset into the recipient; muscle and skin were subsequently sutured with 4-0 absorbable Vicryl.

Post-operative care. Immediately after skin closure, the animals were given Baytril (10mg/kg) and Buprenorphine (0.1mg/kg) subcutaneously. They were then bandaged to prevent access to

the surgical site. The animals were placed in their cage under a heating lamp until fully recovered from the anesthesia. Rats had to be active and mobile before being returned to the animal facility. Bandages were changed every two days and removed on POD7. Grafts were inspected and photographed regularly.

3.2 Machine Perfusion and Settings

The first preliminary experiments served to establish a basic perfusion system to determine if it could successfully perfuse the vasculature and to evaluate the effects of perfusion on the abdominal wall graft. The pump that was utilized was the Ismatec 8-channel peristaltic pump that provided continuous flow. After a graft was harvested and blood removed, a flushing needle connected to the tubing of the pump was inserted a few millimeters into the artery and secured with a silk tie. A small catheter was inserted into the vein to promote an open vessel and allow outflow. Perfusates were prepared as necessary for each experiment and are as follows: (i) HSa: 40mL lactated ringers + 1mL (1,000USP) heparin (ii) HTK with no additions (iii) HTK-albumin: 50mL + 6.25g bovine serum albumin (12.5% albumin). The perfusate was then placed in a reservoir that the pump would draw from. The perfusate would circulate through the graft and any outflow would return to the reservoir. **Figure 6** shows the basic components required for this simple configuration. Perfusion experiments were conducted for 12 hours, after which the tissue was processed for analysis.

The settings of the Ismatec pump allowed for a fixed flow rate or rotations per minute (RPM). Several variables were explored in an attempt to find optimal settings that would allow preservation of the graft. They are summarized in Table 2. Several modifications were made to

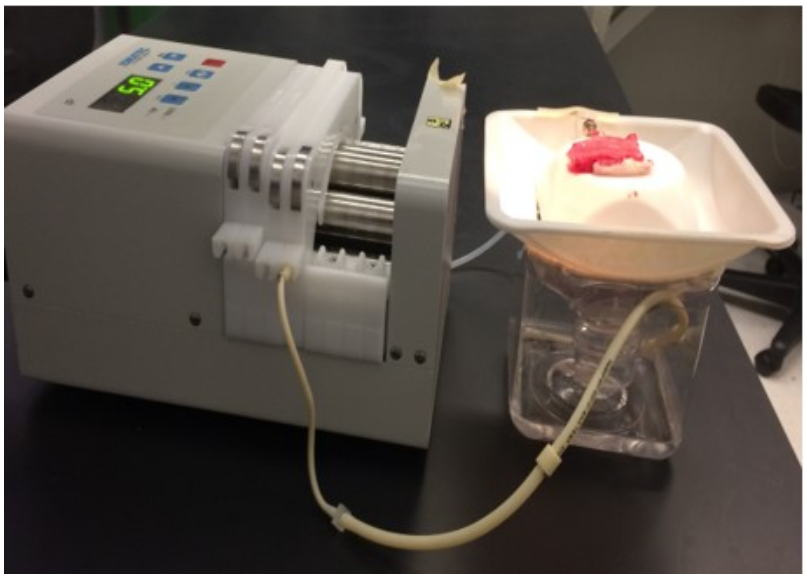

Figure 6. Simple machine perfusion set up. The peristaltic pump draws perfusate from the reservoir and circulates it through the graft. Outflow drains back into the reservoir.

the perfusion system; one of the final prototypes is shown in **Figure 7** with several in-line perfusate sensors, a force sensor, and electrical stimulation capabilities. Additions to the perfusion system, such as a bubble trap, oxygenation, and perfusate heating, are being explored but were not implemented at the time of these experiments.

Table 2. Different variables tested for machine perfusion

Variables		
Perfusate	**Flow Rate**	**Perfusion Type**
HSa	0.5mL/min	Continuous
HTK	0.1mL/min	Intermittent
HTK-albumin		

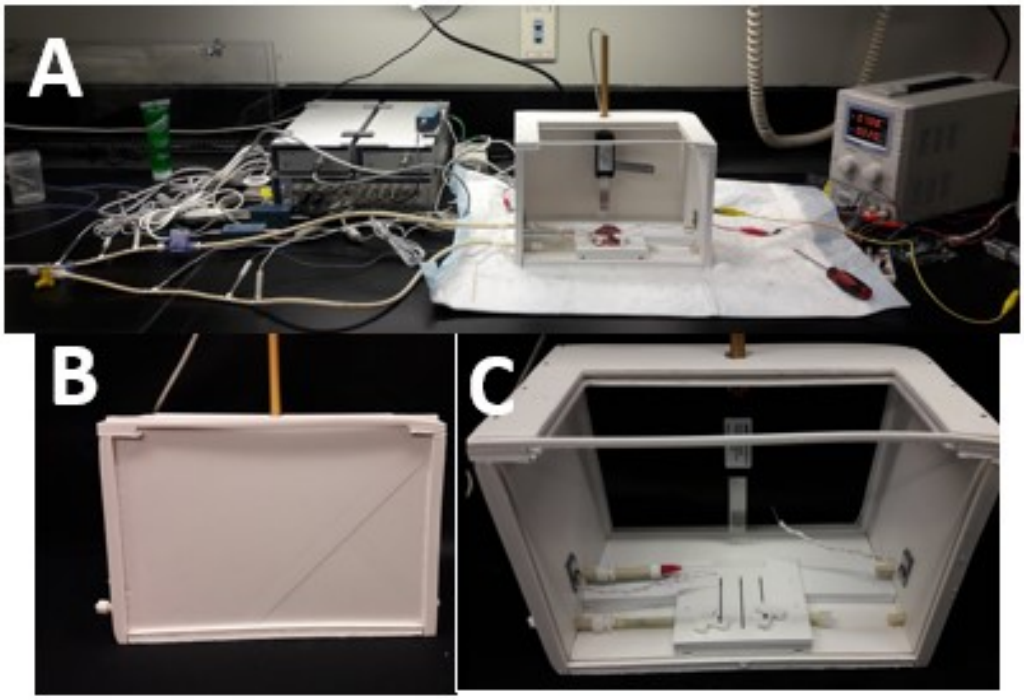

Figure 7. One of the final bioreactor prototypes with necessary components. A) Complete perfusion system. From left to right: tubing from perfusion pump with in-line sensors leading into the bioreactor chamber, data acquisition system, bioreactor chamber. electrical stimulation system B) Front view of bioreactor chamber. C) Inner view of chamber with perfusion ports, tissue clamps, and force sensor visible

3.3 Realization of Bioreactor Housing

When tackling the task of designing the bioreactor housing, it was important to consider the specific needs of the abdominal graft and the transplantation model. The bioreactor housing had to be designed to meet certain criteria, summarized as follows:

- Allow for sterile assembly
- Provide sterile, closed environment for graft perfusion
- Maintain humidity within the chamber to promote graft hydration
- Provide physiological stimulation to maintain muscle function

- Secure graft during perfusion to minimize movement and enable acquisition of force measurements
- Include ports to allow perfusate and external stimuli to be delivered to the graft

To meet these criteria, the design had to include ports to allow access from the outside, clamps to secure the tissue in place, and a component to fix a force sensor. SolidWorks, a computer-aided design (CAD) program, was used to produce prototype models that could be 3D printed and tested before a design was finalized. Prototype printing was carried out on the LulzBot TAZ 5 with PolyLite™ polylactic acid (PLA), which warps and shrinks less than other materials. Multiple iterations of prototypes were created. Modifications were made often, as each use of a prototype would elucidate any shortcomings and improvements that could be implemented.

3.4 Electrical Stimulation Circuit

The electric circuit had to be designed in a way that could stimulate the entire graft at once. To meet this goal, an H-bridge circuit was constructed. This electronic circuit enables a voltage to be applied across a load, which, in this case, is the abdominal wall graft. **Figure 8** shows the circuit diagram of the H-bridge circuit used in these experiments. The components necessary to implement this circuit are illustrated in **Figure 9**. The H-bridge is unique in that it has two different current inputs and channel MOSFETs. Depending on the type of channel MOSFET, applied voltage will open or close a certain pathway. As voltage is applied to either Input A or B, they open and close rapidly, and the direction of current flow through the graft alternates (See **Fig. 7**). This produces biphasic stimulation with alternating current. This is important because monophasic stimulation results in a net ion flow within the tissue which causes ion gradient depletion subsequent tissue damage [71]. The first experiments were to establish proof of

concept and ensure the H-bridge circuit could induce contractions. Voltage was applied between 5-7V. As seen in **Figure 9**, tin-coated 22-gauge copper wires act as electrodes that can be placed on the graft to conduct current. The electrodes were placed on either side of the vascular tree, as shown in **Figure 10**. The tissue was stimulated at 70Hz with 300ms pulse trains every 5 seconds. Several methods were attempted to determine the most vigorous contractile response. Electrodes were placed on the surface of (i) a dry graft (ii) a graft moistened with lactated ringers (iii) a graft fully submerged in lactated ringers or (iv) a graft covered in SignaGel electrode gel. Videos were taken of these experiments and analyzed with a MatLab script to calculate the percentage contraction. After many trials, it was found that applying electrode gel produced the best contractile response with these electrodes and had the added benefit of maintaining graft hydration. Additional experiments were conducted after the graft was connected to the bioreactor's perfusion system. Contraction force in this setting was measured by attaching a small wire into the medial or lateral side of the graft, depending on desired orientation, and attaching the other end to a force sensor, as illustrated in **Figure 11** (ADInstruments MLT1030/D Wide Range Force Transducer). When the graft contracts, a force is applied to the sensor via the wire. The force sensor contains a strain gauge that changes electrical resistance when force is applied. This change can be measured and recorded with a data acquisition system (ADInstruments PowerLab) and correlated to applied force.

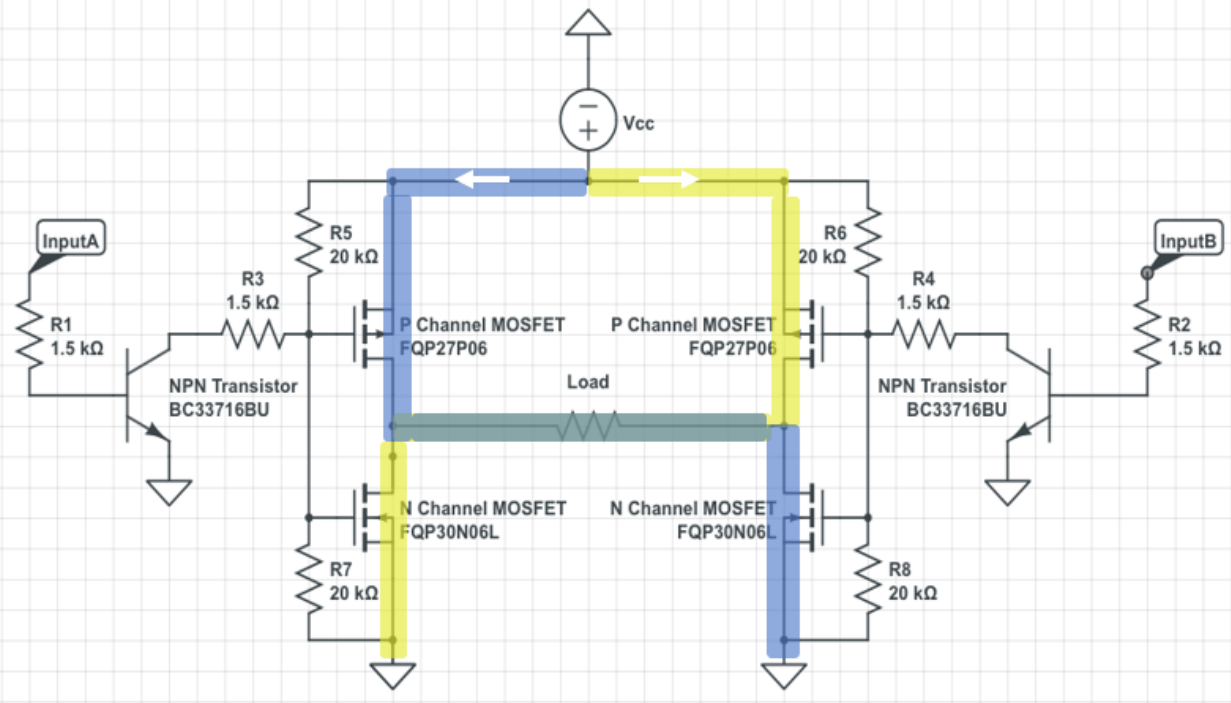

Figure 8. H-Bridge circuit diagram. The circuit consists of two inputs, several resistors, P/N channel MOSFETS, and a voltage source. The resistor labeled "Load" represents the abdominal wall graft. Current is supplied by the voltage source (Vcc). When voltage is applied to Input A, current flows through the path highlighted in yellow. When voltage is applied to Input B, current flows through the path highlighted in blue. They grey area shows overlap where the current stimulates the graft. White arrows indicate direction of current

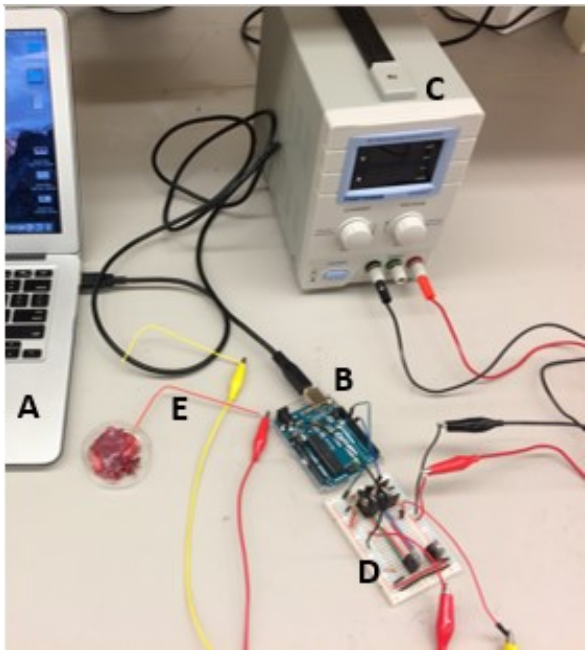

Figure 9. Necessary components for electrical stimulation. A) Laptop B) Arduino microcontroller C) Power supply D) H-bridge circuit built on breadboard E) Platinum electrodes conducting voltage to graft

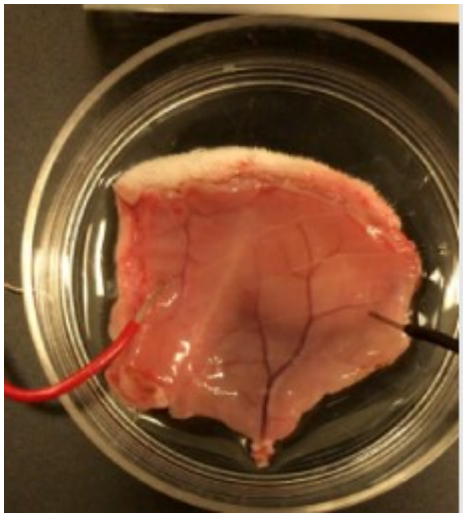

Figure 10. Electrode placement on graft for electrical stimulation

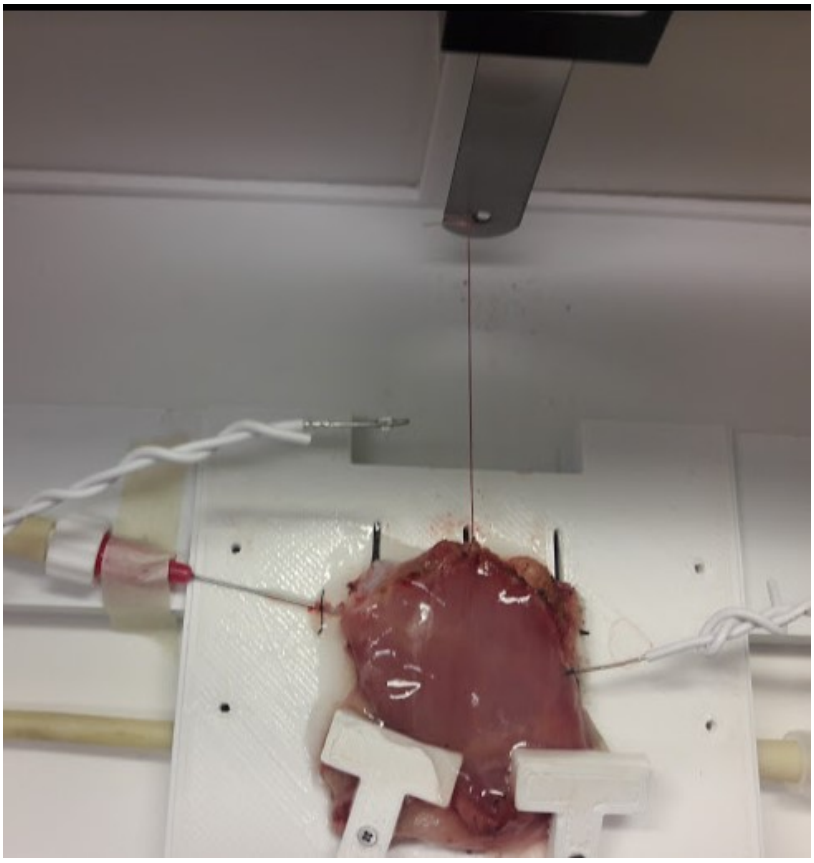

Figure 11. Connection of abdominal wall graft to force sensor. As the graft contracts, the force is conveyed to the force sensor through the attached wire. Clamps are placed as a counter-force for more accurate measurements.

3.5 Implementation and Production of Metabolite Sensors

A variety of sensors were procured or developed to measure different components in the perfusate. These could be important markers of viability and can provide feedback on the metabolic state of the graft throughout the preservation period. In-line sensors were utilized for non-invasive monitoring and were designed for placement in line with the tubing delivering perfusate to the graft. Sensors readily available on the market were procured for oxygen, pH, temperature, and pressure. Other sensors had to be developed for measurement of specific metabolites at low concentrations, including nitric oxide (NO), and hydrogen peroxide (H2O2).

Each sensor was placed both in in-flow and out-flow lines to monitor the graft's response to the circulating perfusate. Each sensor had to be calibrated before every experiment. Table 3 shows physiologic ranges of different parameters in rats that could be used to evaluate graft status.

Table 3, Physiologic ranges of rat parameters

Parameter	Range/Mean	Source
Temperature (°C)	35.9 – 39.4	[76]
pH	7.33 ± 0.07	[77]
Pressure (mmHg)	91 - 129	[78]
P_O2 (mmHg)	95.14 ± 14.42	[77]
Lactate (mM)	1.09 ± 0.28	[79]
Glucose (mg/dL)	60 - 125	[80]

In-line sensors that had to be specially manufactured were produced by the Bioelectronics Laboratory at the University of Maryland, Baltimore County (UMBC). The H2O2 sensor was manufactured with a tungsten wire coated with a gold sheet. In order to increase sensitivity, the sheet was etched with concentrated nitric acid to produce a porous gold surface. The pores increased the surface area, which allowed increased functionalization. The surface was modified by adding 3-Mercaptoproionic acid (MPA), which has a sulfur (thiol) group and a carboxylic acid tail. The thiol groups bond strongly with gold atoms, and the carboxylic acid can be further functionalized. The next step involved conjugating horseradish peroxidase (HRP) to the reactive carboxylic acid tails. HRP catalyzes the reaction that reduces H2O2 into water and an electron. This released electron produces a current that can be detected, measured, and correlated with H2O2 concentration. **Figure 12** summarizes this process. To evaluate the electrode, sensitivity, specificity and stability was determined, and calibration curves were produced. Sensitivity and specificity can be determined by subjecting the electrode to a neutral buffer like PBS and adding known concentrations of H2O2, as well as other molecules such as glucose and acetaminophen. Changes in current indicate the presence of H2O2 and can show if undesired reactions are

occurring with other components in the perfusate. Calibration curves are constructed by evaluating the chronoamperometric response of the electrode. This is done by stepping the potential of the electrode by adding specific volumes of H2O2 in known concentrations in PBS. The resulting current can be monitored as a function of time, and a line of best fit can be extrapolated and used to find H2O2 concentration as a function of current.

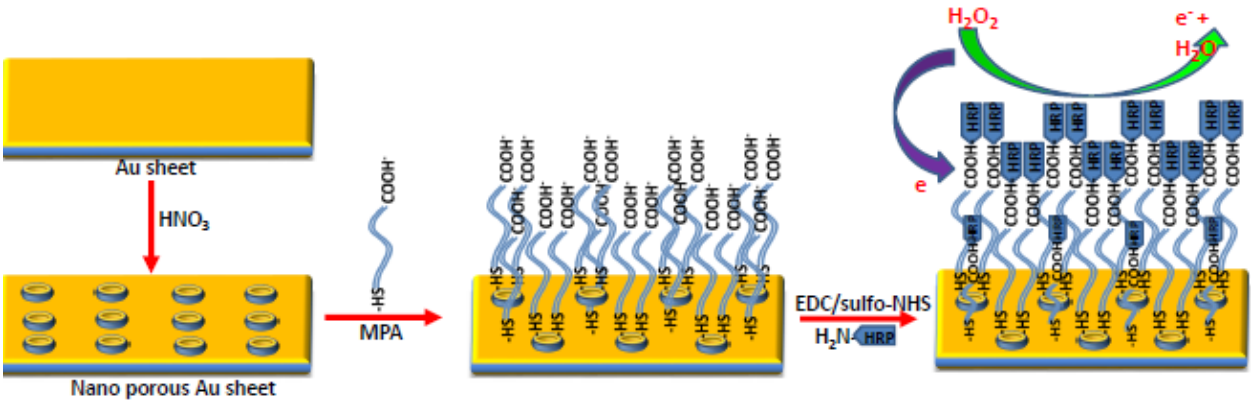

Figure 12. Production of sensing mechanism for H2O2 sensor. Figure provided courtesy of the Bioelectronics Laboratory at (UMBC)

3.6 Tissue Analysis

Samples were taken of the muscle component of the graft after each perfusion experiment to evaluate viability. The tissue was divided into 6 sections, as shown in **Figure 13**. Each section was snap-frozen in isopentane, cryo-sectioned, and stained with hematoxylin and eosin (H&E). Necrosis scores were assigned from 1-4 based on the following criteria: (1) non-homogenous eosinophilic staining (2) vacuole formation (3) hypercontraction (4) macrophage infiltration. Muscle fiber separation was determined as a measure of necrosis. Separation occurs due to rupturing of epimysium and endomysium. It can also indicate pressure-induced damage due to perfusion. Muscle separation was quantified by converting H&E images into binary, black and white images on MatLab, processing the image with a script, and determining the fraction of

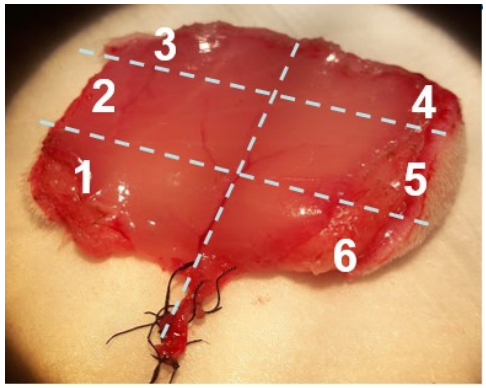

Figure 13. Division of graft muscle for tissue analysis

white areas to black areas. Grafts were weighed before and after perfusion to determine weight gain, which indicate extent of edema.

4. Results

It is important to note that the results reviewed in this section reflect the progress made in the preliminary phases of the project. Progress is continuing as each component of the bioreactor is tested and optimized.

Abdominal Wall Transplant

The procedures conducted during this phase of the project were syngeneic transplants in order to optimize the procedure without the complications of immunosuppressive protocols and possible rejection episodes. Modifications were made to the surgical procedure outlined by Broyles et. al to adapt the transplantation model to the needs of this project. Namely, the graft size was reduced to approximately 3x3cm to maximize perfusion as several previous transplantation attempts found better success with smaller graft sizes. However, trials with securing the graft in the

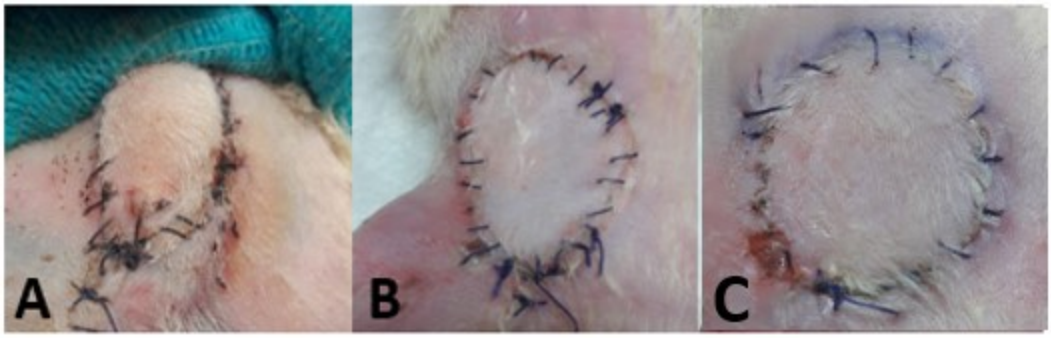

Figure 14. Three recipients with successful syngeneic abdominal wall transplants. A) Recipient 1 on POD3 B) Recipient 2 on POD3 C) Recipient 3 on POD9

bioreactor found that a larger graft size would facilitate the process. The graft was harvested at a larger size so there would be extra tissue to secure clamps and electrodes. Tissue in contact with these elements may sustain damage, so a larger graft size would enable the removal of damaged tissue prior to transplant. This was found to be a feasible course of action by reducing graft size after harvest and immediately before transplant. Although no perfusion time was used in these trials, it was found that reducing graft size after harvest could yield successful transplantations. **Figure 14** shows three successful syngeneic graft recipients on various PODs.

Machine Perfusion Protocols

Initial perfusion experiments involved perfusing methylene blue at 0.5mL/min to ensure that the perfusion system could access the vasculature of the graft. It was seen that the entire graft could be perfused in less than 20 minutes. This is illustrated in Appendix B. Subsequent experiments used HSa as the perfusate at 0.5mL/min for 12 hours at room temperature. This group was compared with two other groups: storage in HSa at room temperature (RT-static) or at 4°C (CS-static) for 12 hours. Necrosis scores were lowest in the CS-static group, and highest in the perfusion group. Muscle fiber separation was the most severe in the perfusion group, and the

grafts experienced an average of 90% weight gain, compared with 30% in RT-static group and 15% in CS-static. **Figure 15** shows representative H&E slides of each group compared with a

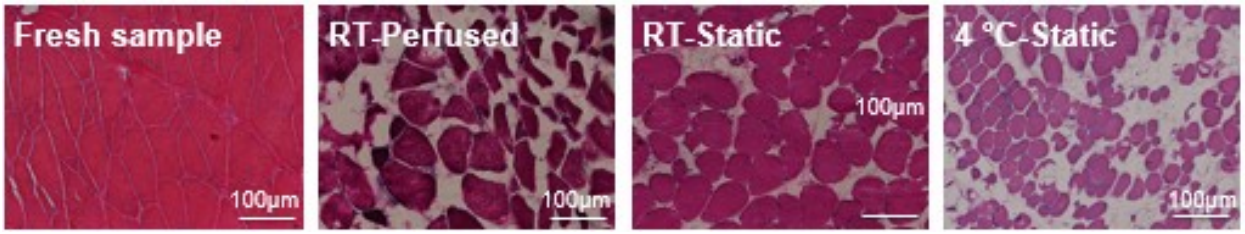

Figure 15. Representative H&E staining of initial perfusion studies with HSa. Loss of cellular structures is evident in all groups

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fresh muscle sample. The disruption of cellular structure is evident in all experimental groups. **Figure 16** shows the calculation of muscle fiber separation; it is clear to see increased white areas in the perfused sample. **Figure 17** is a graphic representation of necrosis scores and fiber separation in each section of the abdominal muscle graft.

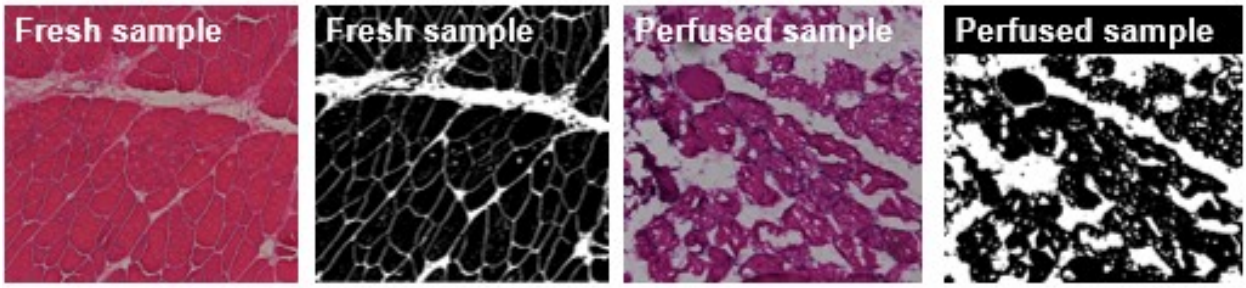

Figure 16. Illustration of how muscle fiber separation is determined. The white area is representative of separation and can be quantified with a computer script. It is clear that the perfused sample has increased white areas, and therefore, increased muscle fiber separation.

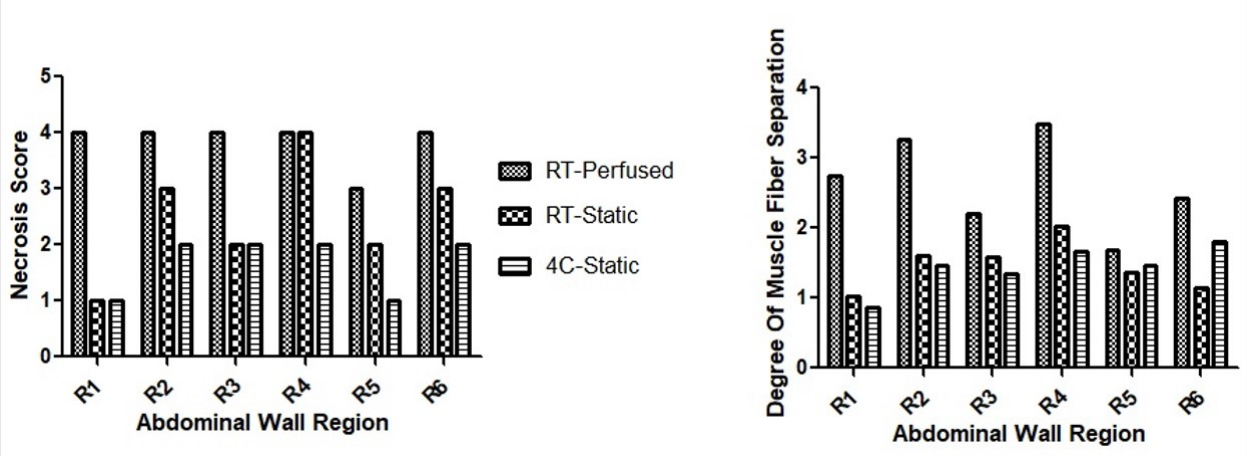

Figure 17. Necrosis scores and degree of muscle fiber separation in perfused and static grafts. The general trend points to room temperature perfusion causing increased necrosis and fiber separation/swelling

In an attempt to reduce swelling, the cauterized edges of the graft were removed to promote more perfusate outflow. The perfusate was also changed to HTK, a common organ preservation solution more suited to maintain balanced cellular components. Implementing these changes decreased swelling and necrosis scores, with an average of 50% weight gain. **Figure 18** illustrates these findings.

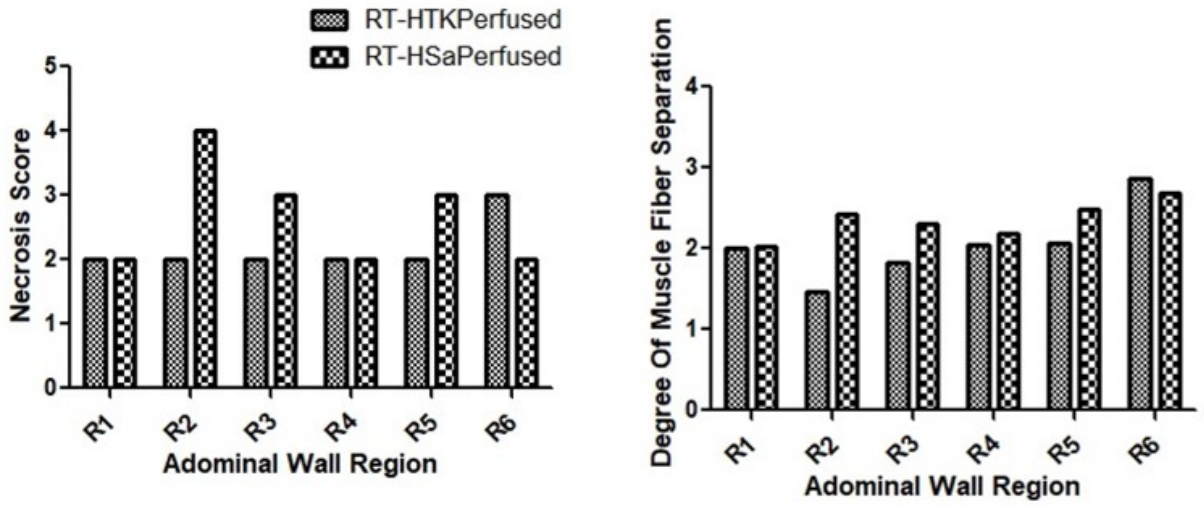

Figure 18. Necrosis scores and degree of muscle fiber separation in grafts perfused with HTK and cauterized edges removed. Necrosis scores and fiber separation were improved in these experiments

To further reduce swelling, an intermittent pattern of perfusion was tested. Instead of 12 hours of continuous perfusion of HTK, the flap was perfused in 20-minute intervals for the first 8 hours, then continuously perfused for the last four. **Figure 19** shows the results of this perfusion pattern. It provided further reduction in pressure induced injury, although necrosis. scores remained unchanged. Additional experiments were carried out by adding 12.5% bovine serum albumin to the HTK solution. Adding this protein to the perfusate increased the oncotic pressure and promoted fluid retention, therefore reducing tissue edema. Weight gain decreased from previous experiments and was approximately 30%. Flow rate was also reduced to 0.1mL/min to further reduce pressure-induced damage. **Figure 20** shows histology of muscle tissue subjected to different perfusion protocols with HTK. It is evident that these protocols reduced edema and muscle separation, but muscle viability was greatly compromised in all experimental samples, with vacuole formation visible in every image. This suggests a need for improved perfusate composition.

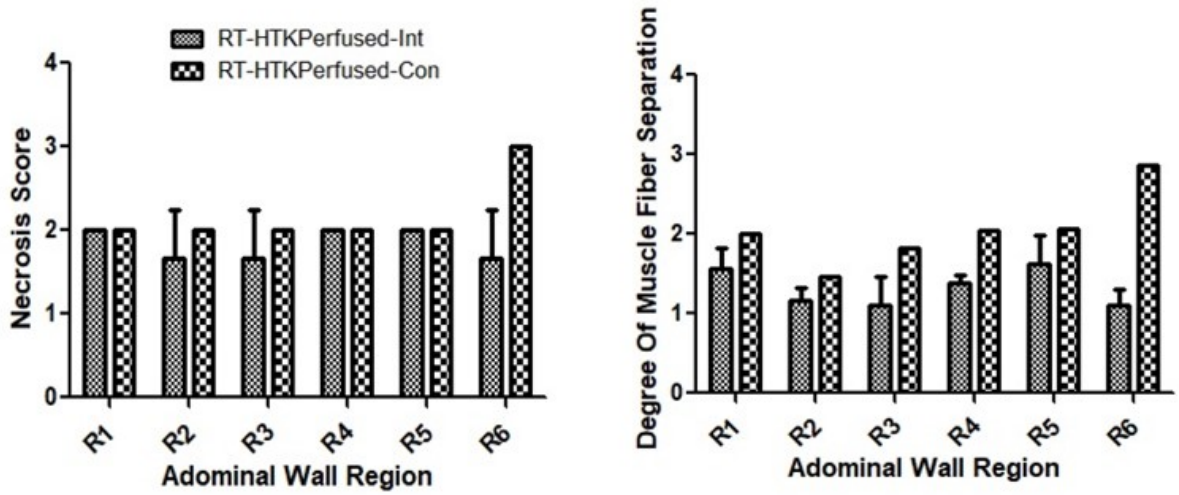

Figure 19. Necrosis scores and degree of muscle fiber separation in intermittent and continuous perfusion with HTK. Fiber separation was reduced, indicating reduced pressure-induced injury and swelling. Necrosis scores were relatively unchanged

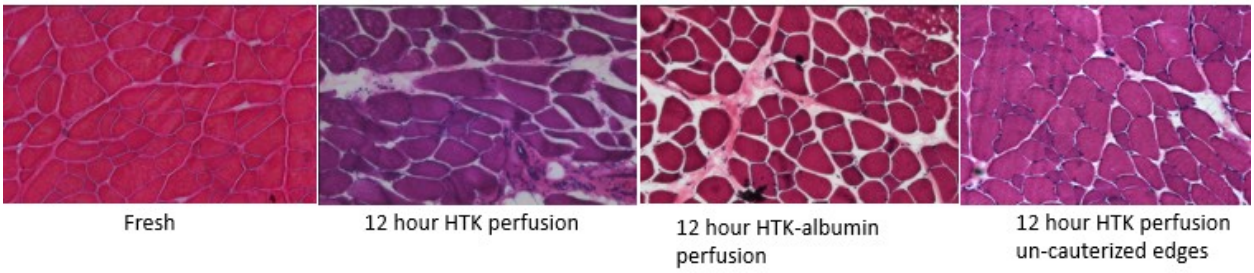

Figure 20. Representative H&E slides of muscle tissue subjected to different perfusion protocols. HTK reduces swelling and pressure-induced damage when compared with HSa, but necrosis scores remained relatively unchanged.

Bioreactor Housing

Prototype models were constructed using SoldWorks software. The main components included in every iteration were: (i) electrode connection tabs for electrical stimulation (ii) tissue mounting plate (iii) tissue clamps (iv) force sensor and (v) perfusion ports. **Figure 21** shows a top-down view of the initial prototype with all these components are labeled. **Figure 22** shows these components in greater detail. The metal tabs allow attachment of alligator clips that allow conduction of electrical stimulations from the external H-bridge to the graft inside the closed bioreactor. The tissue clamps secure the tissue and minimize movement, as well as providing a counter force for the force sensor to acquire data upon contraction. The small slits on the mounting plate allow perfusate to collect in a reservoir underneath the platform, where it can be taken up by the pump for recirculation. For ease of assembly, the bioreactor was constructed with removable sides and roof. This allows the rest of the construct to be permanently

assembled, and the only steps necessary to complete a closed environment are to slide the walls into place (**Fig. 23**).

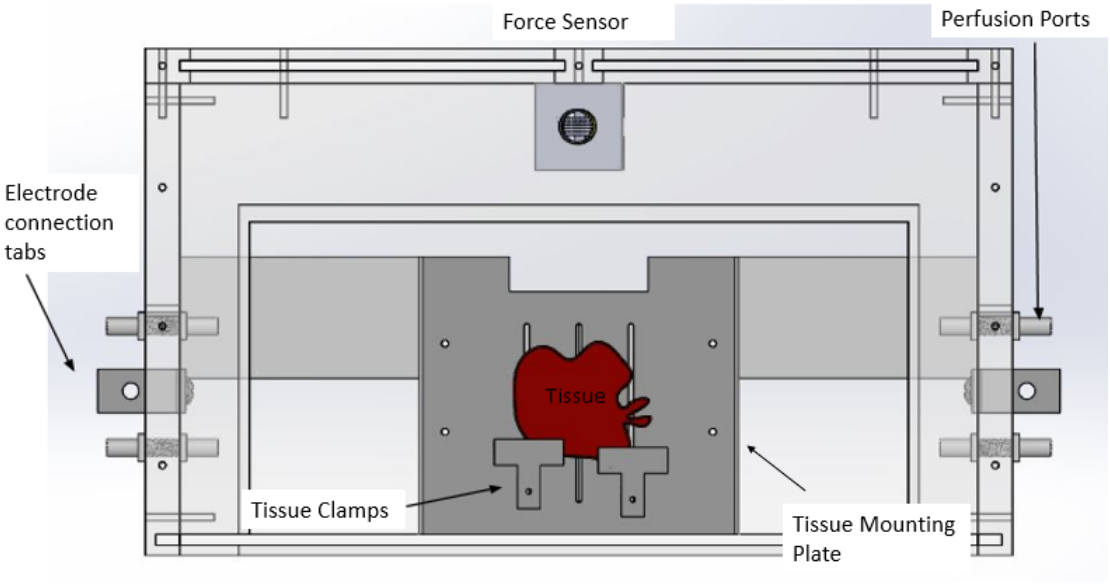

Figure 21. Top-down view of bioreactor SolidWorks model. The necessary components for perfusion and electrical stimulation are labeled

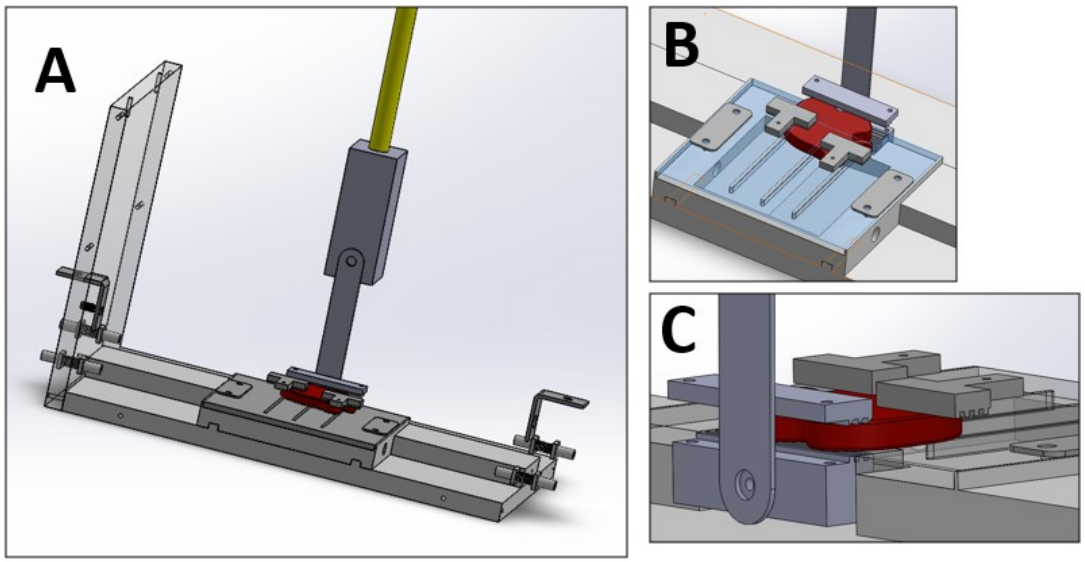

Figure 22. Close-up views of bioreactor components. A) Broad view showing force sensor (yellow), electrical stimulation tabs, and tissue mounting plate B) Tissue mounting plate and tissue clamps C) Tissue clamps and where force sensor connects with the tissue

Every individual component, including removable walls, was printed on the LulzBot TAZ 5 and fully assembled before use. **Figure 24** shows the bioreactor prototype after 3D printing and assembly, and how the graft can be placed on the tissue mounting plate and connected to the perfusion ports.

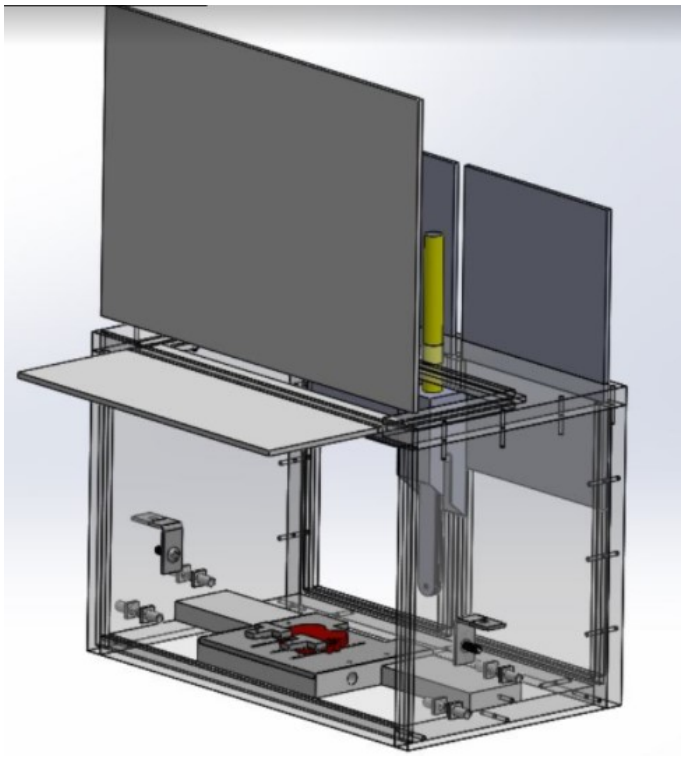

Figure 23. Modification of bioreactor prototype for ease of assembly. Sides are roof are removable. Assembly is complete once these components are slid into place

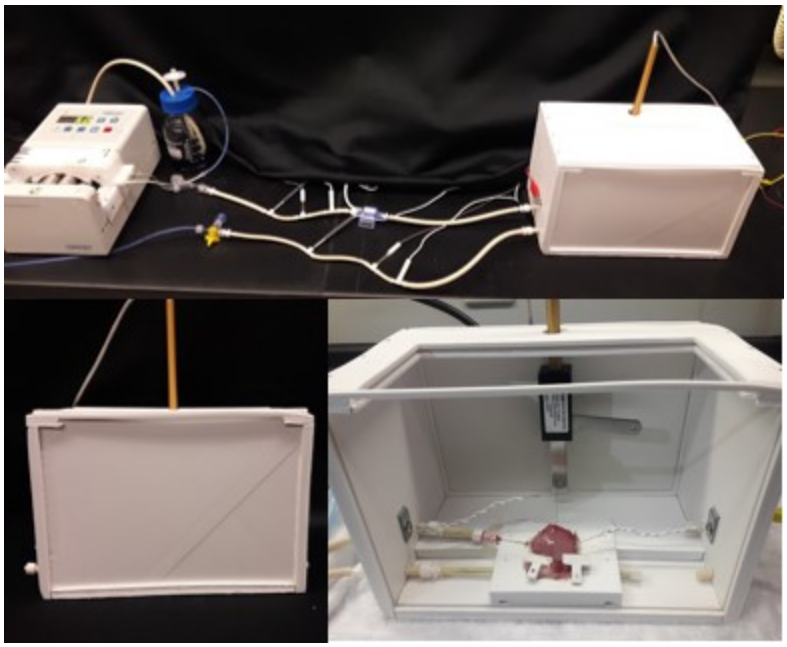

Figure 24. Assembled prototype and connection to abdominal wall graft. A) Perfusion system with peristaltic pump (left) delivery perfusate through tubing to bioreactor (right) B) Outside view of bioreactor chamber C) Inside view of chamber with perfusion needle attached to artery

Electrical Stimulation

It was found that applying SignaGel to the surface of the graft was the most effective in inducing a vigorous muscular contraction. **Figure 25** shows placement of electrodes on gel-coated tissue, and the graft before and after contraction.

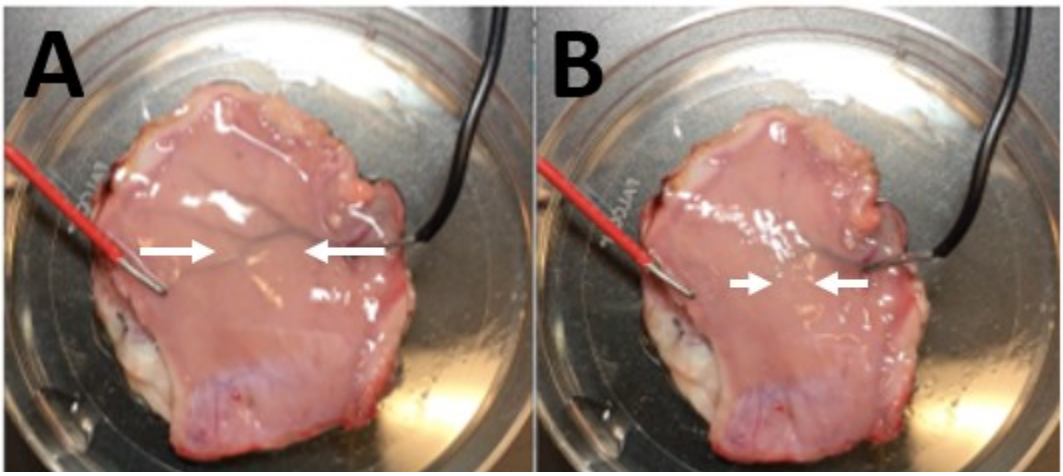

Figure 25. Electrical stimulation induces muscular contraction. A) Muscle at rest, before stimulation B) Muscle contracting during stimulation. White arrows indicate direction of contraction

Fatigue tests showed that both 5-second and 2-minute intervals between applied stimulation fatigued the muscle before six hours post-surgery (**Fig. 26**). With 5 second intervals, the percent contraction (change in graft length) was lower than with 2-minute intervals, and contractions ceased at an earlier time point. Although electrical stimulation of the muscle was achieved, no force measurements were acquired. The force sensor needs further calibration and the bioreactor chamber needs further modifications to better enable force measurements.

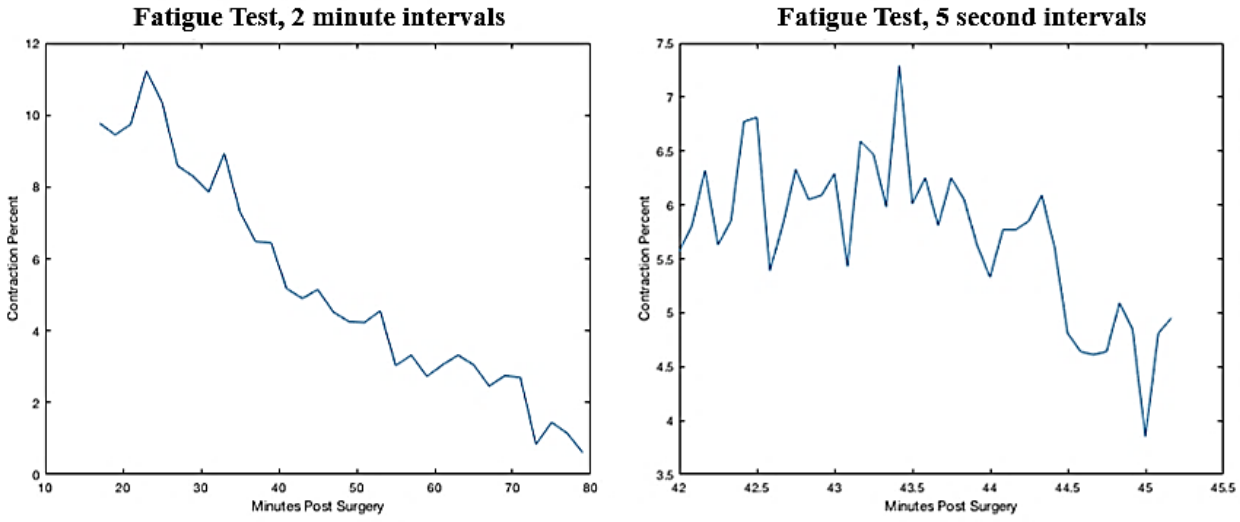

Figure 26. Fatigue test for 2-minute and 5-second intervals in electrical stimulation. Response to stimulation gradually reduced until no contraction was observed.

Metabolite Sensors

The Tungsten-wire based H2O2 electrode was found to have a linear dynamic range of 800pM to 5mM, which is wide enough to detect the physiologic ranges found in most organisms, including rats. **Figure 27** shows the chronoamperometric response of the electrode to H2O2 at different

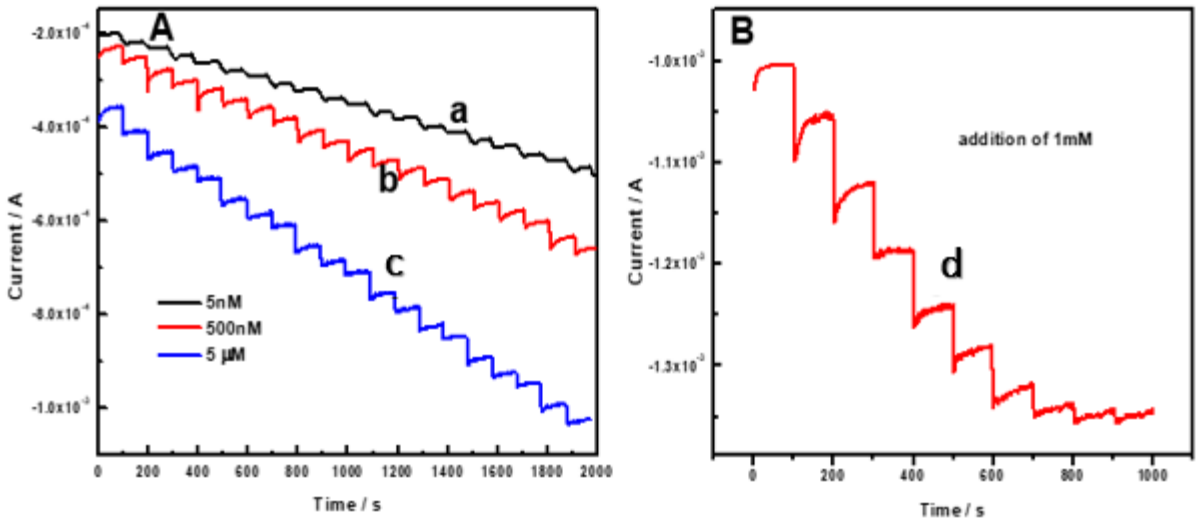

Figure 27. Chronoamperometric response of H2O2 electrode at different concentrations. A) Responses to 5nM (a), 500nM (b), 5uM (c) of H2O2. B) Response to 1mM (d) H2O2. This shows fast response behavior and allows for subsequent calibration curves. Figure provided courtesy of the Bioelectronics Laboratory at UMBC

concentrations. Responses were within 3 seconds, indicating an electrode with fast response behavior. **Figure 28** shows the calibration curves extrapolated from these responses.

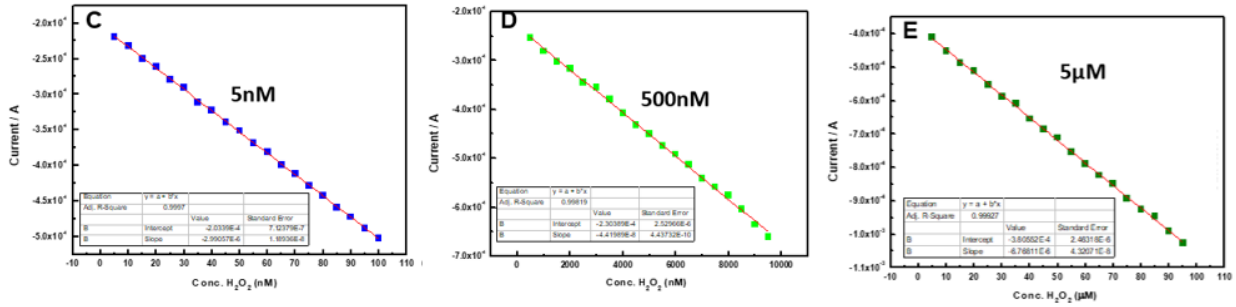

Figure 28. Steady-state current-time responses of Tungsten electrode to different H2O2 concentrations. This yields a wide linear range of sensitivity from 800uM to 5mM. Figure provided courtesy of the Bioelectronics Laboratory at UMBC

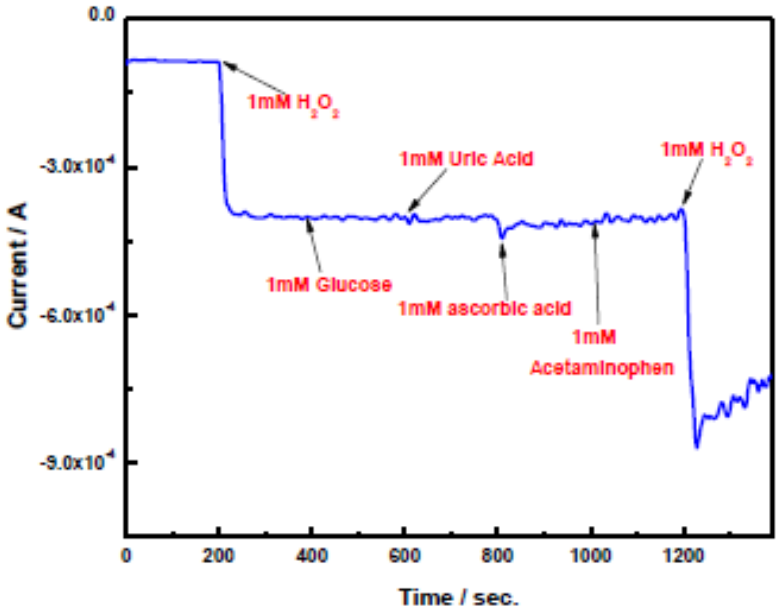

Figure 29. Selectivity of H2O2 Tungsten electrode. Additions of glucose, uric acid, ascorbic acid, and acetaminophen do not induce a change in current. This shows that the electrode is sensitive to H2O2 only. Figure provided courtesy of the Bioelectronics Laboratory at UMBC

The reduction currents of H2O2 were linear in the range of 800pM to 5mM, with a correlation coefficient of 0.99. This linear range covers the physiological concentrations known for H2O2. The specificity of the sensor was determined by adding interfering species to the buffer. Current was only increased with the addition of H2O2, and none of the other species (**Fig. 29**).

Other sensors that were employed were able to detect pressure, temperature, and percent oxygen in in-flow and out-flow perfusate. **Figure 30** shows examples of the data acquired by these sensors.

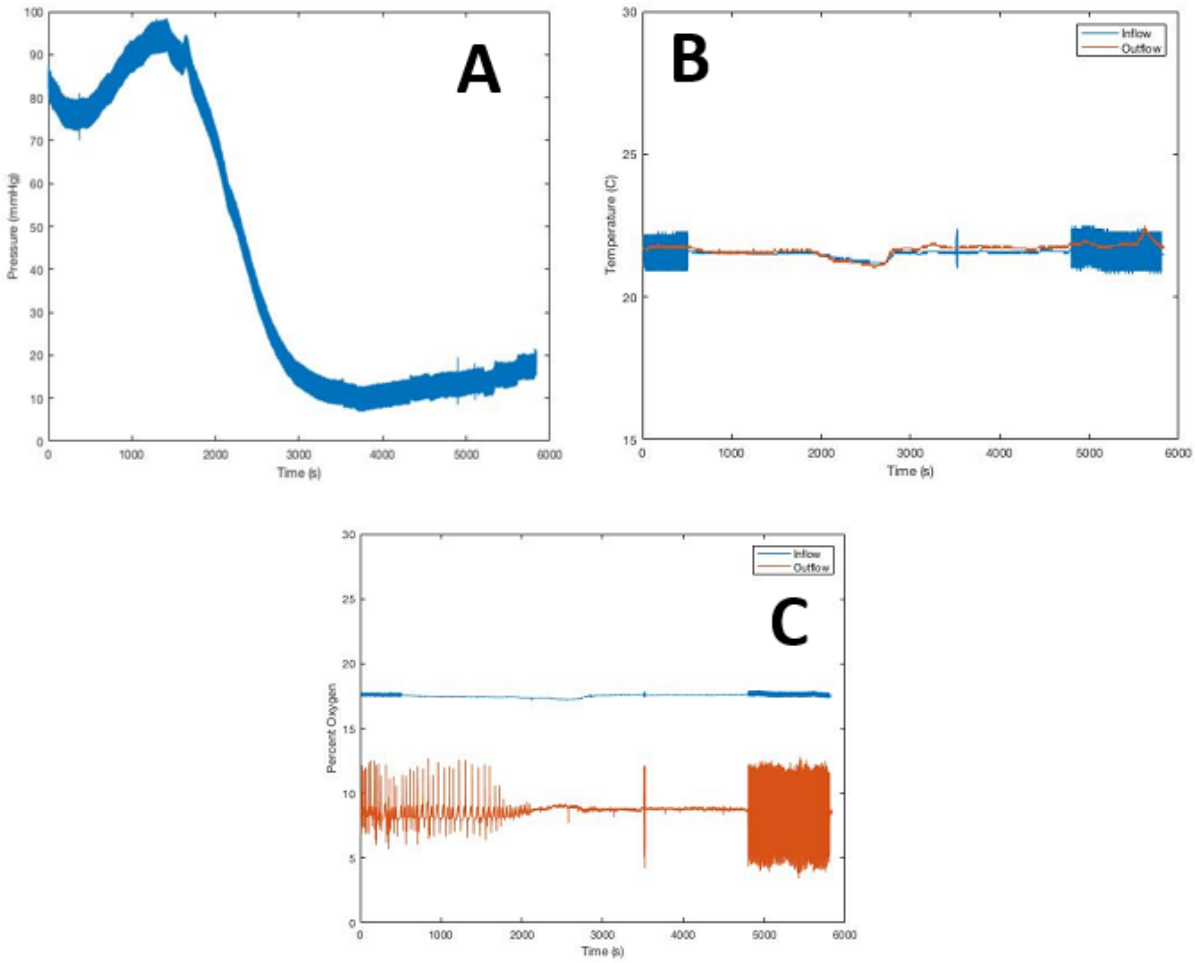

Figure 30. Sample data acquired from in-line sensors during perfusion experiments. A) Pressure readings B) Temperature reading; blue indicates inflow; orange indicates outflow C) Oxygen content readings; blue indicates inflow, orange, outflow

5. Discussion

The murine abdominal wall transplant model was established as successful, technically feasible, and reproducible in our lab. Daily monitoring is important to observe any mechanical trauma caused by auto-mutilation, and to take appropriate steps to prevent further trauma to the graft. Additional transplants must be completed in order to monitor graft survival and animal behavior past POD10.

Bioreactor prototypes can be effectively produced using SolidWorks models and 3D printing techniques. Each component can be individually printed and assembled prior to use. Changes in the structure can be made to existing SolidWorks models to improve iterations of the prototype. The latest prototype is built for ease of assembly. Further prototypes will include spring-loaded clamps for simpler securing of the tissue, rubber sealing to ensure a closed environment for optimal humidity and sterility, and the implementation of an arterial catheter to reduce potential damage to the artery.

The perfusion system can be set up with in line sensors for pH, oxygen, temperature, and pressure. Calibration of each sensor prior to each experiment ensures more consistent data throughout. Each sensor in the in-flow and out-flow pathways can provide real-time data through the data acquisition system. The rate of the perfusion pump can be fixed in order to control pressure throughout the perfusion. Rates of both 0.5mL/min and 0.1mL/min are effective in perfusing the vasculature of the graft in a short amount of time, but pressures must be monitored, and rate can be adjusted to maintain pressure within a certain range. It was evident in early experiments that HSa perfusion caused more damage to the graft than static preservation, regardless of storage temperature. Although HTK reduced edema when compared to HSa, it was still unacceptable with approximately 40% weight gain. Albumin was added to increase oncotic

pressure and encourage fluid retention. This helped to reduce swelling to approximately 30%, so there is still room for improvement. Removing the cauterized edges of the graft also helped to relieve swelling, but this is not translatable to future transplantation procedures as un-cauterized edges would bleed freely following reestablishment of perfusion. There were not enough perfusion trials to reach statistical significance but observing general trends during the preliminary phase of this project allowed modifications in methods while reducing excessive use of resources. Future experiments require further additions to the perfusate, namely oxygen carriers, glucose, vasodilators, and antibiotics. These additions will allow for better maintenance of metabolism and graft viability. The sensors will be integral in determining optimal additions of each component.

The electrical stimulation circuit was able to induce muscular contractions in the graft, although contraction force decreased over time. In an ideal environment, the muscle would be receiving enough nutrients to replenish energy stores and waste removal to prevent accumulation of toxic end-products. This scenario would, ideally, prevent or diminish muscle fatigue and allow muscular contractions to occur throughout the 12-hour preservation period. Unfortunately, the force sensor was unable to gather meaningful data, as it needs further calibration and additional modifications within the tissue mounting plate for proper function. Intervals at which stimulation should be applied must be determined, although our fatigue tests suggest intervals of greater than 10 minutes to maintain more uniform contractions.

The H2O2 sensors can reliably and effectively measure H2O2 concentrations, even in the presence of interfering species. However, this function was determined in a neutral buffer, so further experiments will require the use of the perfusate used in perfusion experiments. By testing H2O2 detection in this solution, we can determine whether or not the sensor's function is

the same in our experimental perfusate. Once this sensor is finalized, it will be able to measure H2O2 concentration and correlate it to presence of ROS to evaluate tissue damage and ischemia. Sensors will also need to be manufactured to detect nitric oxide and lactate, for further determination of tissue viability.

In conclusion, our experiments have shown that our machine perfusion system can perfuse the vasculature of the murine abdominal wall graft, much in the same way as solid organ perfusion systems can. Although the basic principles behind solid organ machine perfusion can be applied to VCA perfusion, it is important to understand that the vasculature must be maintained in order to perfuse the different components of the graft. For example, different vessels may perfuse the skin and fat layers, while others perfuse the muscle. Constructing a custom bioreactor greatly facilitates the process of connecting the graft to the perfusion system, while also enabling electrical stimulation and perfusate monitoring. The perfusates used thus far, however, are insufficient to extend graft survival. However, as this project is in its very early phases, there is still much experimentation needed to determine the optimal conditions for successful preservation of the abdominal wall graft. This includes pressure settings, temperature settings, perfusate composition, and viability marker profiles. It is clear that the perfusate is one of the most important components that must be optimized for enhanced graft preservation. Future work is also needed to acquire contraction force data, as well as H2O2 data, in conjunction with 12-hour perfusion experiments.

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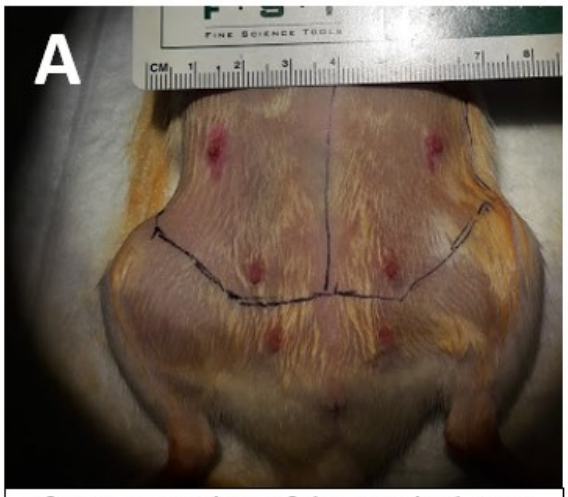
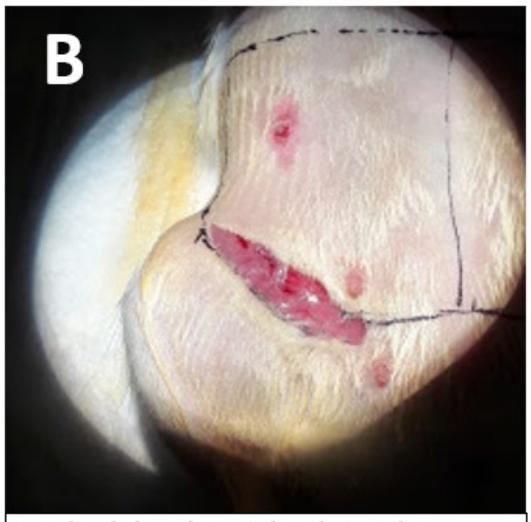
Appendix A: Surgical Procedures

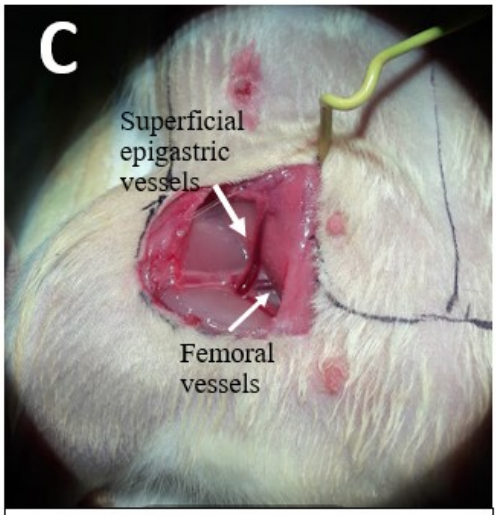
Abdominal Wall Harvest



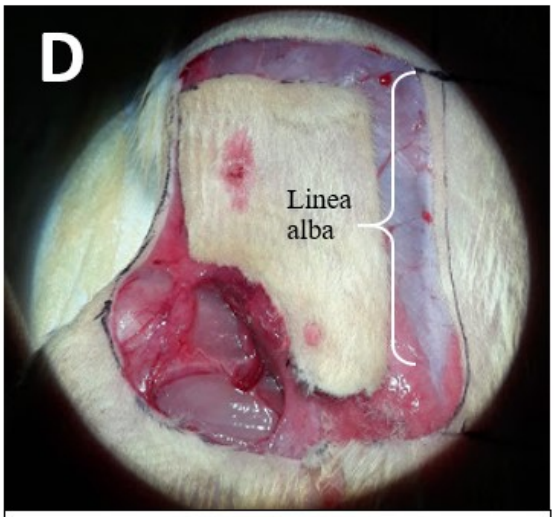
After preparation of the surgical area, an area of approximately 4x4cm is marked on either side of the linea alba



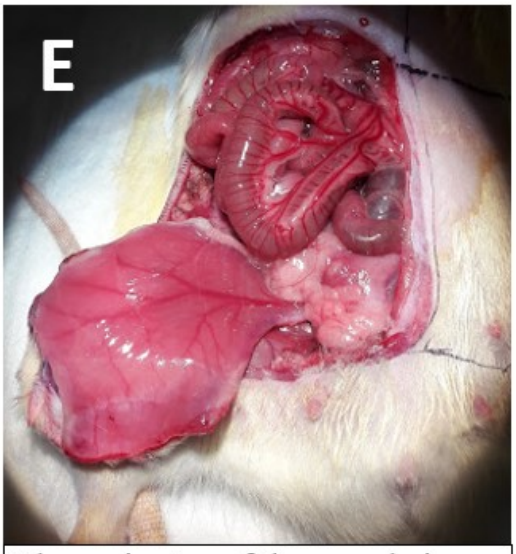
An incision is made along the inguinal canal to and fascia dissected to expose superficial epigastric and femoral vessels



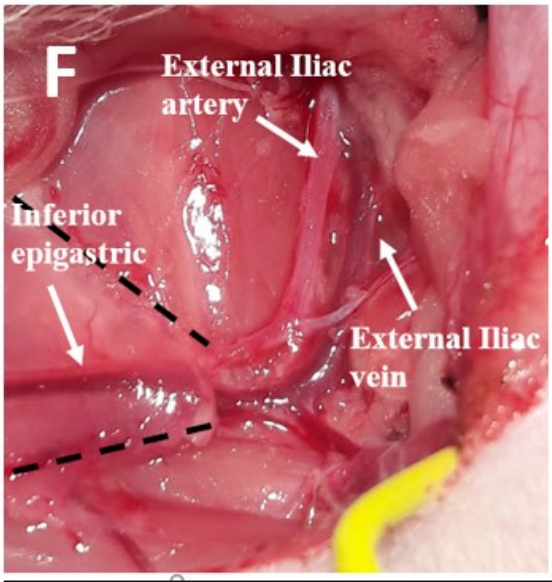
The superficial epigastric and femoral vessels are identified and dissected



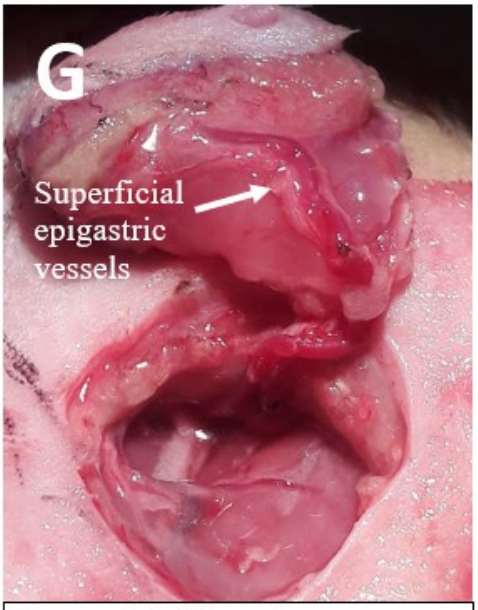
The skin is cut around the graft perimeter. The linea alba is clearly visible



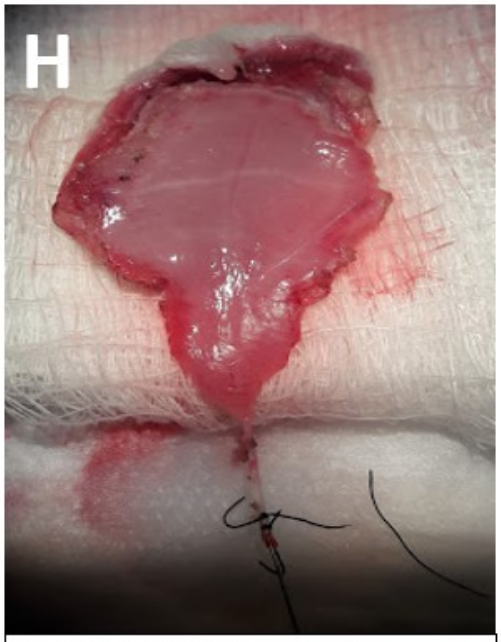
The perimeter of the muscle is cauterized and everted caudally



Organs are retracted to expose the external iliac vessels. The black dotted line represents the borders of the graft

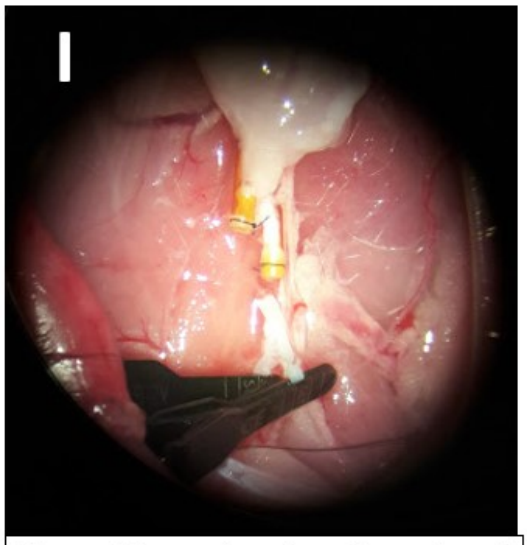


The graft is flipped to access and dissect the femoral vessels. They are divided distal to the superficial epigastric

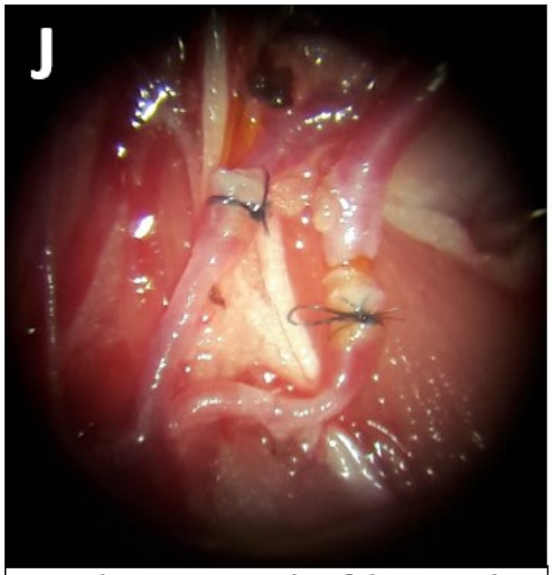


The graft is removed and a flushing needle inserted in the artery to remove blood with HSa

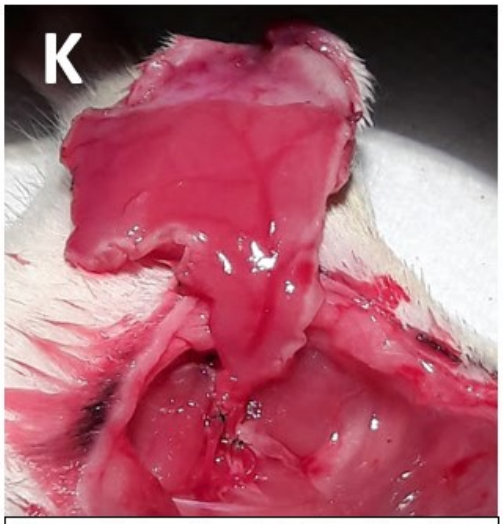
Abdominal Wall Transplantation



The cuffs are placed on the external iliac vessels for subsequent anastomosis



Vascular anastomosis of donor and recipient vessels is completed

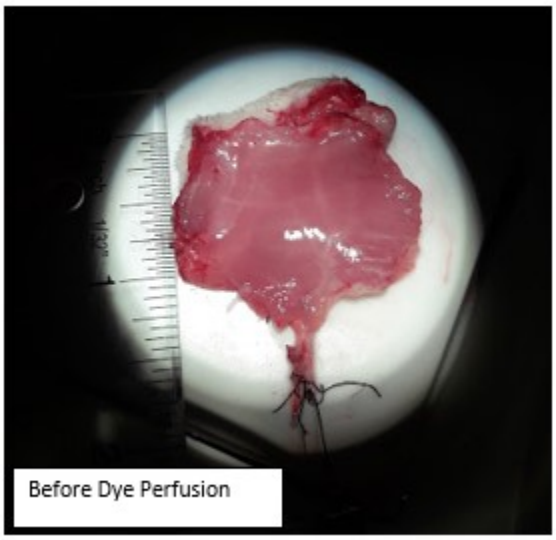


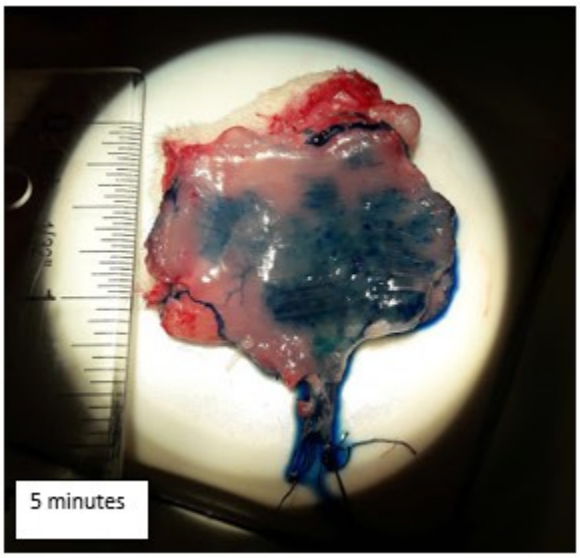
Reperfusion is established and graft returns to normal, healthy color. Recipient abdominal wall is removed, and graft inset

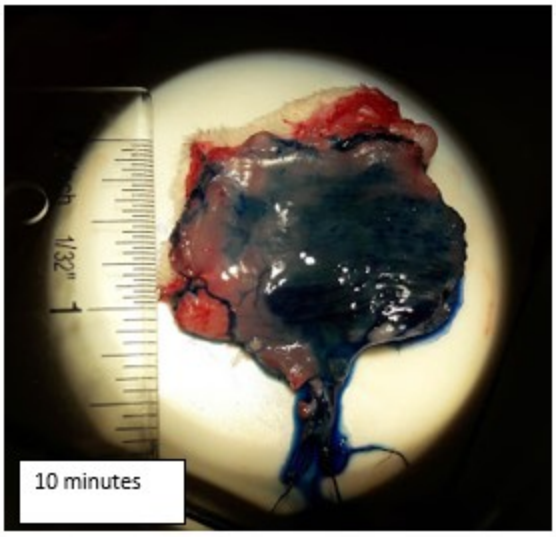
Muscle and skin are sutured and animal is allowed to recover

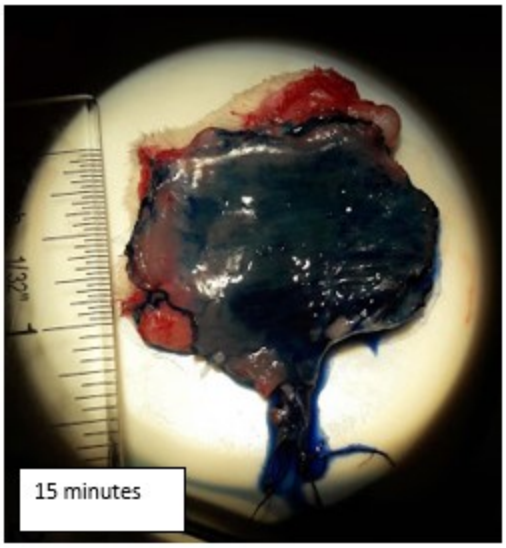
Appendix B: Perfusion Experiments

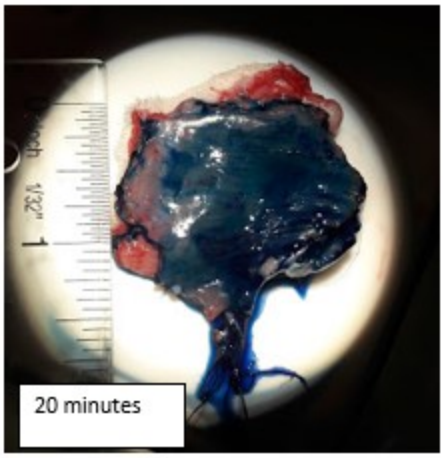
Methylene Blue Dye Perfusion










20 minutes

Curriculum Vitae

Vanessa Guarnizo

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EDUCATION

Master of Science in Biomedical Engineering Anticipated completion: May 2018
Johns Hopkins University, Baltimore, Maryland (GPA: 3.54/4.00)

- Relevant Coursework: Systems Bioengineering 1 & 3, Cellular Engineering, Chemistry of Materials Synthesis, Biomedical Applications of Glycoengineering

Bachelor of Science in Biomedical Engineering May 2016
University of Florida, Honors College, Gainesville, Florida GPA (3.81/4.00)

- Relevant Coursework: Cell & Systems Physiology, Circuits I, Introduction to Biochemistry and Molecular Biology, Biomedical Instrumentation, Cell Engineering Lab

RESEARCH EXPERIENCE

Vascularized Composite Allotransplantation Research Laboratory, Baltimore, Maryland February 2017-Present
Graduate Research Fellow

- Conduct various surgical techniques in rats, including vascular anastomosis and abdominal wall harvest and transplantation
- Design, construct and implement novel bioreactor for functional preservation of composite tissue grafts
- Evaluate efficacy of immunomodulatory drugs delivered via localized hydrogels
- Prepare blood and serum samples for flow cytometry and detection of cytokines using magnetic bead panels

University of Florida Department of Materials Science & Engineering, Gainesville, Florida
Biomaterials Research January 2015-May 2016

- Mimicked natural bone formation in human molars, collagen sponges, and rat tail tendon using the polymer-induced liquid-precursor (PILP) process
- Prepared samples for scanning electron microscopy, transmission electron microscopy, and x-ray diffraction

WORK EXPERIENCE

Milton S. Eisenhower Library, Baltimore, Maryland August 2016-May 2017
Technical Services Graduate Student Assistant

- Catalogued large volumes of books and completed various projects in detail-oriented manner
- Physically processed delicate and rare books

Center for Leadership and Service, Gainesville, Florida August 2015- May 2016
Student Assistant

- Coordinated programs such as MentorUF and Florida Alternative Breaks in collaboration with staff
- Communicated with staff to build and maintain a productive work environment

University of Florida Housing Office, Gainesville, Florida May 2014-May 2015
Customer Service Representative

- Managed high call volumes and visitors; acted as liaison between students/families and staff
- Trained and mentored new employees
- Led weekly housing tour groups of up to 50 individuals
- Translated for Spanish speaking visitors

INVOLVEMENT

Biomedical Engineering Society, University of Florida August 2013-May 2016
Peer Advisor

- Organized events designed to increase awareness of the biomedical field in collaboration with members
- Provided peer advising to fellow BME students through individual and group advising sessions

Community Health Service Corps, University of Florida January 2015-May 2016

- Organized Relay for Life and other outreach events such as the Migrant Farm Workers Health Fair

TECHNICAL SKILLS

- Proficient in Microsoft Word, Excel, PowerPoint
- Familiar with MATLAB and C++
- Fluent in Spanish